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GENETIC DISSECTION OF RESISTANCE TO WESTERN GALL RUST (*ENDOCRONARTIUM HARKNESSII* (J.P.MOORE) Y. HIRATSUKA) IN LODGEPOLE PINE (*PINUS CONTORTA* VAR. *LATIFOLIA* ENGELM)

by

CHANGXI LI

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

In

FOREST BIOLOGY AND MANAGEMENT

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **GENETIC DISSECTION OF RESISTANCE TO WESTERN GALL RUST (*ENDOCRONARTIUM HARKNESSII* (J.P.MOORE) Y. HIRATSUKA) IN LODGEPOLE PINE (*PINUS CONTORTA* VAR. *LATIFOLIA* ENGELM)** submitted by **CHANGXI LI** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **FOREST BIOLOGY AND MANAGEMENT.**

Dr. Francis C. Yeh (supervisor)

Dr. Yasu Hiratsuka

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Date September 28 / 1994

ABSTRACT

Geographic variability in western gall rust (WGR) (*Endocronartium harknessii* (J.P.Moore) Y. Hiratsuka) sampled from lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.) host at four locations in British Columbia and Alberta, and jack pine (*Pinus banksiana* Lamb.) host at nine locations in Alberta, Saskatchewan, Manitoba and Ontario was investigated using 41 random amplified DNA markers (RAPDs). Eighteen RAPDs were polymorphic, of which 15 could discriminate between WGR isolates of lodgepole and jack pine hosts. Of these 15 RAPDs, six and four were unique to the isolates from lodgepole pine and jack pine, respectively. The remaining five were significantly heterogeneous ($P \leq 0.01$) in RAPD counts between the hosts. RAPD pattern in lodgepole pine was uniform while that in jack pine differed among locations with an east-west trend of decreasing affinity. Analysis of molecular variance apportioned 76.3%, 14.4% and 9.3% of total RAPD variability to differences among hosts, among locations within hosts and with locations, respectively. The large differentiation between WGR fungal isolates from lodgepole pine and jack pine hosts might suggest the selective pressure for host specificity in sampled populations was strong.

Association between WGR resistance and RAPDs was investigated in three lodgepole pine open-pollinated families (A00588, A01013 and A01754) that showed significant variation in WGR resistance in field trials. A framework RAPD linkage map was constructed using a LOD score of 3.0 to group the markers for each family using megagametophytes. The corresponding embryos were challenged in the greenhouse against two WGR isolates, one each from lodgepole pine and jack pine hosts for QTL

identification. Linkage map of A00588 has 225 RAPDs in 16 groups, and covers 3517.5 cM. Linkage map of A01013 has 172 RAPDs in 16 groups, and covers 3496.0 cM. Linkage map of A01754 has 234 RAPDs in 17 groups, and covers 3398.2 cM. Eight putative RAPDs are common across the three maps, and on average, 34 putative RAPDs are common between two maps. The linkage relationships of the common putative RAPDs were conservative, but the order and distance between the linked putative common pairs varied.

Fourteen putative QTLs for resistance to WGR were mapped with LOD scores that ranged from 3.86 to 9.37. Family A00588 has three QTLs on linkage group 9. Two each explained 35.6% and 32.8% and jointly 40.2%, of total phenotypic variance in resistance to WGR from lodgepole pine, and the remaining one explained 23.2% of the total phenotypic variance in resistance to WGR from jack pine. Family A01013 has nine QTLs in six linkage groups. Five QTLs from linkage 1, 7, 10 and 11 each explained 82.6% to 86.5% and jointly 87.4% of the total phenotypic variance in resistance to WGR from lodgepole pine. Four QTLs from linkage groups 10, 13 and 16 each explained 81.7% to 82.6% and jointly 84.2% of the total phenotypic variance in resistance to WGR from jack pine. Family A01754 has two QTLs on linkage groups 13 and 3 that explained 87.3% and 81.5% of the total phenotypic variance in resistance to WGR from lodgepole pine and jack pine, respectively. Results suggest that resistance to WGR in lodgepole pine involve genes of large effect. It should be noted that 5 of the 14 QTLs had LOD scores less than 4.90. Given that fact the progeny size was 30 and 45 to test the two rust sources, they have a false positive rate of about 1×10^{-3}.

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TABLE OF CONTENTS

CHAPTER 1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION 1

1.1.1 Background of western gall rust 1

1.1.1.1 Occurrence and distribution of western gall rust 1

1.1.1.2 Life cycle of western gall rust fungus 3

1.1.1.3 Disease cycle and damage caused by western gall rust 5

1.1.2 Background of lodgepole pine (*Pinus contorta* Dougl.) 6

1.1.2.1 Species and distribution 6

1.1.2.2 Commercial status of lodgepole pine 7

1.1.2.3 The incidence of western gall rust in lodgepole pine 8

1.1.3 Traditional methods in control of western gall rust in hard pines 8

1.1.3.1 Removal of infected trees and branches 9

1.1.3.2 Chemical control method 9

1.1.3.3 Biological control methods 10

1.1.3.4 Screening resistance to the disease 11

1.1.4 Manipulation of both the rust and the host 11

1.1.4.1 General concept of manipulation of both the rust and the host 12

1.1.4.2 Strategies and prerequsites toward the effective manipulation of both the rust and the host 12

1.2 LITERATURE REVIEW AND DISCUSSION 13

1.2.1 Genetic studies in host-pathogen pathosystem 13

1.2.1.1 General terms in describing host-pathogen interaction 14

1.2.1.2 Molecular markers in genetic study of host-pathogen pathosystem 15

1.2.1.3 Development of bulk segregation analysis (BSA) and its application in identification of molecular markers linked to resistance genes in host-pathogen pathosystem 17

1.2.1.4 Development of QTL analysis and its application in identification of molecular markers linked to resistance genes in host-pathogen pathosystem 18

1.2.2 Genetic studies in the western gall rust and lodgepole pine pathosystem 23

1.2.2.1 Genetic variability in western gall rust fungus 23

1.2.2.2 Resistance variation in lodgepole pine 25

1.2.2.3 Western gall rust and lodgepole pine interaction 27

1.2.2.4 The identification of genetic markers linked to resistance to western gall rust in lodgepole pine and other hard pine species 30

1.3 STUDY OBJECTIVES AND GENERAL EXPERIMENTAL DESIGN..... 31
1.3.1 Study objectives..... 31
1.3.2 General experimental design..... 32

1.4 REFERENCES..... 33

CHAPTER 2 RANDOM AMPLIFIED POLYMORPHIC DNA VARIABILITY AMONG GEOGRAPHIC ISOLATES OF WESTERN GALL RUST FUNGUS

2.1 INTRODUCTION..... 51

2.2 MATERIALS AND METHODS.....52
2.2.1 Collection of fungal spores 52
2.2.2 DNA extraction..... 53
2.2.3 DNA amplification..... 54
2.2.4 Data analysis..... 55

2.3 RESULTS..... 56

2.4 DISCUSSION..... 58

2.5 REFERENCES..... 62

CHAPTER 3 EVALUATION OF RESISTANCE IN THREE ALBERTA LODGEPOLE PINE FAMILIES AGAINST TWO DIFFERENT WESTERN GALL RUST SPORE SOURCES IN GREENHOUSE

3.1 INTRODUCTION..... 77

3.2 MATERIALS AND METHODS..... 78
3.2.1 Isolates collection..... 78
3.2.2 Plant materials..... 79
3.2.3 Experimental design..... 80
3.2.4 Inoculation procedures..... 80
3.2.5 Evaluation of disease infections..... 81
3.2.6 Statistical analysis..... 81

3.3 RESULTS..... 82
3.3.1 Family effect..... 82
3.3.2 Spore source effect..... 83
3.3.3 Family by spore source interaction..... 83

3.4 DISCUSSION.....84

3.5 REFERENCES.....87

CHAPTER 4 CONSTRUCTION OF HIGH DENSITY GENOMIC MAPS IN THREE LODGEPOLE PINE FAMILIES USING RANDOM AMPLIFIED PLOYMORPHIC DNA (RAPD) MARKERS

4.1 INTRODUCTION.....94

4.2 MATERIALS AND METHODS.....97
4.2.1 Plant materials.....97
4.2.2 DNA extraction.....97
4.2.3 DNA amplification.....98
4.2.4 RAPD marker analysis.....99
4.2.5 Linkage analysis and map construction.....100

4.3 RESULTS.....100
4.3.1 Genomic mapping in family A00588.....100
4.3.2 Genomic mapping in family A01013.....101
4.3.3 Genomic mapping in family A01754.....102
4.3.4 Comparison of genomic maps across the three families.....103

4.4 DISCUSSION.....105

4.5 REFERENCES.....110

CHAPTER 5 IDENTIFICATION OF QUANTITATIVE TRAIT LOCI FOR RESISTANCE TO WESTERN GALL RUST IN ALBERTA LODGEPOLE PINE

5.1 INTRODUCTION.....137

5.2 MATERIALS AND METHODS.....139
5.2.1 Plant material.....139
5.2.2 Artificial inoculation and resistance evaluation.....140
5.2.3 Genomic map construction.....141
5.2.4 QTL identification.....142

5.3 RESULTS.....142
5.3.1 QTLs in family A00588.....143
5.3.2 QTLs in family A01013.....144

5.3.3 QTLs in family A01754..... 145
5.3.4 QTL associations among families..... 145

5.4 DISCUSSION..... 146

5.5 REFERENCES..... 153

CHAPTER 6 GENERAL DISCUSSION, CONCLUSION AND IMPLICATIONS

6.1 INTRODUCTION..... 165

6.2 GENETIC VARIABILITY OF WESTERN GALL RUST FUNGUS..... 166

6.3 RESISTANCE OF LODGEPOLE PINE TO WESTERN GALL RUST..... 167

6.4 GENOMIC MAP AND QUANTITATIVE TRAIT LOCI FOR RESISTANCE TO WESTERN GALL RUST IN LODGEPOLE PINE..... 168

6.5 FUTURE STUDIES RECOMMENDED..... 172

6.6 REFERENCES..... 174

LIST OF TABLES

Table 2-1 Collection of western gall rust isolates from jack pine and lodgepole pine hosts..... 68

Table 2-2 Count of variable RAPDs detected in western gall rust isolates in jack pine and lodgepole pine hosts..... 69

Table 2-3 Count of variable RAPDs detected among geographic isolates of western gall rust fungus in jack pine host..... 70

Table 2-4 Analysis of RAPD variance among geographic isolates of western gall rust fungus in jack pine and lodgepole pine..... 71

Table 2-5 Analysis of RAPD variance among geographic isolates of western gall rust fungus in jack pine host..... 72

Table 3-1 Degrees of freedom and expected mean squares in analysis of variance to determine the spore source and family effects (mixed model)..... 90

Table 3-2 Analysis of variance of spore source and family effects based on the $\log_{10}$ (1+disease rating) values..... 91

Table 3-3 The means of disease rating values of three lodgepole pine families across two spore sources..... 92

Table 4-1 Putative common markers in three lodgepole pine families..... 117

Table 5-1 Putative QTLs for resistance to western gall rust in lodgepole pine family A00588..... 159

Table 5-2 Putative QTLs for resistance to western gall rust in lodgepole pine family A01013..... 160

Table 5-3 Putative QTLs for resistance to western gall rust in lodgepole pine family A01754..... 161

LIST OF FIGURES

Figure 2-1 Geographic locations of western gall rust isolates collected from lodgepole pine (L1-L4) and jack pine (J1-L9) across Western and Central Canada..... 73

Figure 2-2 RAPD profiles of western gall rust isolates amplified with Operon primer OPA07 (A) and UBC primer UBC290 (B). Lane 1 and lane 28: molecular weight marker VI (Boehringer Mannheim). Lane 2-lane 9: RAPDs of isolates from lodgepole pine. Lane 10-lane 27: RAPDs of isolates from jack pine. Arrows indicate polymorphic RAPD fragments with their molecular sizes in base pairs on the right..... 74

Figure 2-3 Dendrogram depicting RAPD similarity among geographic isolates of western gall rust fungus in jack pine and lodgepole pine hosts. 75

Figure 2-4 Dendrograms depicting similarity of western gall rust fungus isolates among locations based on cluster analysis of RAPDs amplified by primers from University of British Columbia Biotechnology Laboratory (UBC) and Operon Technologies Inc (Operon)..... 76

Figure 3-1 Distribution of disease scores in lodgepole pine family A00588, family A01013 and family A01754 against western gall rust from Alberta and Manitoba sources..... 93

Figure 4-1 RAPD profiles of DNAs from megagametophytes of lodgepole pine half-sib families. Lane 1: DNA molecular weight marker VI (Boehringer Mannheim). Lane 2 -lane 31: RAPDs of family A00588 amplified by primer Uelj (A), RAPDs of family A01013 amplified by primer Ulef (B), and RAPDs of family A01754 amplified by primer OGer (C). The arrows indicate the segregating fragments with their molecular sizes in base pairs on the right..... 118

Figure 4-2 Linkage map for lodgepole pine family A00588 based on 225 RAPD markers. Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked..... 119

Figure 4-3 Linkage map for lodgepole pine family A00588 based on 225 RAPD markers (continued). Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan

distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked..... 120

Figure 4-4 Linkage map for lodgepole pine family A01013 based on 172 RAPD markers. Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked..... 121

Figure 4-5 Linkage map for lodgepole pine family A01013 based on 172 RAPD markers (continued). Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked..... 122

Figure 4-6 Linkage map for lodgepole pine family A01754 based on 243 RAPD markers. Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked..... 123

Figure 4-7 Linkage map for lodgepole pine family A01754 based on 243 RAPD markers (continued). Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked..... 124

Figure 4-8 Putative parallel linkage group 1 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 125

Figure 4-9 Putative parallel linkage group 2 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 126

Figure 4-10 Putative parallel linkage group 3 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 127

Figure 4-11 Putative parallel linkage group 4 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 128

Figure 4-12 Putative parallel linkage group 5 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 129

Figure 4-13 Putative parallel linkage group 6 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 130

Figure 4-14 Putative parallel linkage group 7 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 131

Figure 4-15 Putative parallel linkage group 8 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 132

Figure 4-16 Putative parallel linkage group 9 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 133

Figure 4-17 Putative parallel linkage group 10 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 134

Figure 4-18 Putative parallel linkage group 11 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 135

Figure 4-19 Putative parallel linkage group 12 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families..... 136

Figure 5-1 Putative QTLs for resistance to western gall rust in lodgepole pine family A00588. The arrow keys and bars indicated the most likely position of the QTL and their corresponding support intervals at LOD of 1.0..... 162

Figure 5-2 Putative QTLs for resistance to western gall rust in lodgepole pine family A01013. The arrow keys and bars indicated the most likely position of the QTL and their corresponding support intervals at LOD of 1.0..... 163

Figure 5-3 Putative QTLs for resistance to western gall rust in lodgepole pine family A01754. The arrow keys and bars indicated the most likely position of the QTL and their corresponding support intervals at LOD of 1.0..... 164

CHAPTER 1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Western gall rust (WGR) is the most common, most conspicuous, and most destructive stem rust of hard pines in western Canada (Ziller 1974). During the last several decades, the biology and distribution of WGR was well documented. Damage by WGR in the hard pine natural stands and plantations has also been frequently reported (Baranyay and Stevenson 1964; Calson 1969; Peterson 1971; Powell and Hiratsuka 1973; Hiratsuka and Powell 1976; Hiratsuka 1981; Bella 1985; Burnes *et al.* 1988).

Lodgepole pine is an important reforestation species in North America, but high incidence of WGR in lodgepole pine stands, especially in young plantations, makes the rust a threatening agent in lodgepole pine management.

Traditionally, efforts to control WGR either focus on the rust or on the host. However, as forest management becomes intensive, the simultaneous manipulation of both the rust and the host should be considered the goal of future research.

Understanding the rust, the host and their relationship is one of the prerequisites toward the effective manipulation of the rust and the host. This chapter will outline the relevant background of both WGR and lodgepole pine. Traditional methods in controlling WGR will also be discussed. Strategies and prerequisites for the effective manipulation of both the rust and the host will be proposed.

1.1.1 Background of western gall rust

1.1.1.1 Occurrence and distribution of western gall rust

Western gall rust disease is caused by a rust fungus *Endocronartium harknessii* (J.P.Moore) Y. Hiratsuka (=*Peridermium harknessii* J.P.Moore). The rust fungus *E. harknessii* is found across North America, from Nova Scotia to the Yukon, in the east southward through New York, Pennsylvania, Western Virginia, and Virginia; and in the west southward to Arizona and northern Mexico (Hiratsuka 1987). The hosts of the fungus in North America involve both native and exotic hard pine species, including *Pinus banksiana* Lamb., *P. contorta* Dougl., *P. mugo* Turra, *P. muricata* D. Don, *P. nigra* Arnold, *P. pinaster* Ait., *P. ponderosa* Laws., *Pinus.* D. Don, and *P. sylvestris* L. (Ziller 1974; Hiratsuka 1987). In Canada, the major hosts of this rust are jack pine (*Pinus banksiana* Lamb.), lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.) and ponderosa pine (*Pinus ponderosa* Laws.) (Hiratsuka 1987).

The most apparent symptom of infection by WGR fungus *E. harknessii* is the conspicuous perennial globose galls produced on the stems of hard pines. In the past, WGR had often been confused with eastern gall rust (*Cronartium quercuum* (Berk.) Miyabe ex Shirai) because eastern gall rust also produces globose galls on its hosts. However, the two rusts are different species and can be distinguished by certain characteristics such as germ-tube development and nuclear behavior during spore germination (Anderson and French 1965; Hiratsuka and Maruyama 1968; Hiratsuka 1969). The germ tube length of eastern gall rust fungus is three times that of WGR fungus when spores are germinated on 2% water agar at 18.5^0 C (Anderson and French 1965). Moreover, WGR fungus is considered to be a pine to pine rust while the eastern gall rust is a heteroecious rust with oak (*Quercus* spp.) as the alternative host. The distribution of both rusts overlaps to some extent and the presence of both rusts has been reported on a

single jack pine tree in northern Minnesota (Dietrich *et al.* 1985). However, WGR is common in Canada while eastern gall rust is rare and probably does not exist in the prairie provinces (Hiratsuka 1987).

In 1926, York reported the occurrence of "Woodgate *Peridermium*" on *Pinus sylvestris* in eastern Northern America (York 1926) and it had been also confused with *Cronartium quercuum* (True 1938; Boyce 1943). Later the rust was considered to be WGR (Boyce 1957; Hiratsuka and Maruyama 1968; van Sickle and Newell 1968).

1.1.1.2 Life cycle of western gall rust fungus

Western gall rust fungus *Endocronartium harknessii* was first described by J.P. Moore in 1876 as *Peridermium harknessii* on *Pinus radiata* and later reported by Hedgcock in 1912 on *Pinus Jeffreyi* (Hopkin 1986). The true life cycle of the rust fungus, however, was not well understood at that time and it was considered to be in the imperfect state. Consequently, the rust fungus was placed in the genus *Peridermium* and included in the *Cronartium coleosporioides* Arth. complex since then (Arthur 1934; Boyce 1943; Cummins 1962).

Studies towards understanding the true life cycle of WGR fungus have been frequent during the last several decades. As early as in 1916, the rust was suggested to exhibit pine to pine infection (Fromme 1916) and was then known as an autoecious rust fungus.

Some researchers have reported that the rust fungus infected *Castilleja miniata* Dougl. as the alternative host (Weir and Hubert 1917; Meinecke 1920; Meinecke 1929). But the facultative heteroecism of the rust fungus was subsequently doubted (Zalasky and Riley 1963; Wagener 1964; Ouellette 1965). Wagener (1964) suggested that the surface

contamination of *Castilleja miniata* by *Peridermium stalactiforme* Arth. and Kern (= *Cronartium coleosporioides*) might contribute to the apparent heteroecism observed by Meinecke (1929). Inoculation tests and cytological studies later indicated that the fungus was purely autoecious (Zalasky and Riley 1963; Wagener 1964; Ouellette 1965; Hiratsuka *et al.* 1966).

The nuclear behavior of WGR fungus spores upon germination was well studied and described by Hiratsuka *et al.* (1966). Based on their observations, Hiratsuka *et al.* (1966) stated that the rust fungus nuclear fusion in the spores and meiosis upon germination of the spores produced septated germ tubes that are considered as basidia although no basidiospores are produced. Due to its endocyclic life cycle nature of its autoecism, the new genus *Endocronartium* was erected by Hiratsuka in 1969 to accommodate the rust and other endo species having morphological similarities to the imperfect genus *Peridermium* and species with aecioid telia of the *Cronartium* type (Hiratsuka 1969). The WGR fungus was then named as *Endocronartium harknessii* (J.P.Moore) Y. Hiratsuka.

Although later some other reports claimed that the nuclear division of the aeciospores was mitotic (Christenson 1968; Laundon 1976; Epstein and Buurlage 1988; Vogler *et al.* 1997), it is well accepted now that the rust fungus *E. harknessii* is an endocyclic, autoecious rust, possessing only one spore state and no alternate host (Allen *et al.* 1990b). Based on the observations by Hiratsuka (1991a) and Hiratsuka *et al.* (1966), the life cycle of *E. harknessii* can be described as follows.

Aeciospores of *E. harknessii* are produced on the galls and can infect the green tissue of young shoots directly. Immature spores usually have two nuclei, while the mature spores just before germination have one nucleus due to the nuclear fusion. Upon

germination the spores produce germ tubes and subsequent meiotic nuclear division results in 2 to 4 uninucleates in the germ tube. Germ tubes usually divide into three, four or five cells separated by septa and the growth is determinate. Each segment of a septate germ tube usually has one nucleus. The germ tubes often have side branches that are functional and are involved in host penetration. Dikaryotization of the monokaryotic and haploid hyphae then take place at the base of sorus and the dikaryotic cells divide to produce the spores that annually sporulate on the gall surface.

1.1.1.3 Disease cycle and damage caused by western gall rust

Powdery, orange-yellow aeciospores (aecial teliospores or peridermioid teliospores) of *E. harknessii* are produced on the surface of galls from May to June (Peterson 1973; Hiratsuka 1987). The spores are airborne and can infect new young shoot tissues of the hard pines directly. The spores of *E. harknessii* germinate between a temperature of 10-30 ^{0}C with the optimum germination occurring between 15 and 20 ^{0}C (Powell and Morf 1966). After germination, the germ tubes of the spore germlings grow either perpendicular or parallel to the epidermal ridges of the hypocotyles (Hopkin *et al.* 1988). Once the monokaryotic and haploid hyphae reach the cambium, they stimulate repeated division of the cambial cells, causing the production of excess xylem and ray parenchyma (Allen *et al.* 1990a; Peterson 1960, Hiratsuka and Powell 1976), resulting in the gall formations 1-2 years after infection. Galls grow each year and produce spores every spring for many years, unless the gall tissue dies with the stem or the sori are inactivated by mycoparasites (Hiratsuka 1987).

The galls produced on the main stems of young trees often kill the trees. Trees with the main stem galls tend to be deformed and broken easily at the gall, resulting in mortality (van der Kamp 1989) or the deformity prevents the trees from reaching merchantable size (Blenis *et al.* 1988). Branch infections usually serve as major sources of new infections and have little impact on the growth of lodgepole pine. However, an unusually high abundance of branch infections in lodgepole pine can also result in mortality, or affect the growth significantly. The impact of the disease is not great in the natural forests. However, as forest management becomes more intensive, the damages will likely increase in young pine plantations (Allen *et al.* 1990b). Serious damage by WGR in the young hard pine plantations and the nurseries has been observed in disease surveys (Powell and Hiratsuka 1973; Hiratsuka 1981).

1.1.2 Background of lodgepole pine (*Pinus contorta* Dougl.)

1.1.2.1 Species and distribution

Lodgepole pine is the most widely distributed conifer species in western Northern America. The natural range of its distribution, centered in British Columbia, spans about 33^0 of latitude from Yukon down to Southern California, 35^0 of longitude from the Pacific coast to the center of Colorado and 3900 m of elevation (Wheeler and Critchfield 1985).

Lodgepole pine is remarkably uniform in chromosomes. It is a diploid species and has 12 pairs of large chromosomes (Critchfield 1980). However, lodgepole pine demonstrates great geographic variation in the morphological, physiological, and biochemical traits over its vast distribution range, and has been generally recognized as being comprised of

three geographic subspecies, spp. *contorta*, spp. l*atifolia*, spp. *murrayana* and one edaphic ecotype, spp. *bolanderi* (Wheeler and Critchfield 1985). In the coastal region, subspecies *contorta* is sometimes called the shore pine. The subspecies occupies a variety of extreme habitats along the coast. Subspecies *murrayana* is commonly found in the southern Cascades, Sierra Nevada and the mountains of southern Baja California and is a minor component of the Sierra Nevada subapline forest (Wheeler and Critchfield 1985). spp. *bolanderi* occurs in the Mendocino White plains along the northern California coast. The taxonomic status of this population however, is still questionable (Wheeler and Critchfield 1985). Subspecies *latifolia* is the most widely distributed, ranging along the Rocky Mountain region, from the Yukon to Colorado and eastern Oregon. This subspecies is commonly known as the lodgepole pine. The name lodgepole pine hereafter referred to in this thesis is subspecies *latifolia.*

1.1.2.2 Commercial status of lodgepole pine

Lodgepole pine is one of the major forest resources in western North America, covering over 26,000,000 ha of the forestland (Critchfield 1980). For the last several decades, lodgepole pine has been one of the most important reforestation species in North America because of its desirable silvicultural traits, including good wood quality, relatively short rotation, and the ability to grow under diverse site conditions. In British Columbia, lodgepole pine is the number one species both in harvesting and planting, comprising one-quarter of the total harvest and 35% of total planting (B.C. Ministry of Forests 1992). In Alberta, lodgepole pine is the second most important commercial forest species after white spruce (*Picea glauca*), occupying 22.3% of the forests and accounting

for about 35% of the merchantable timber volume (Dhir and Barnhardt 1993). The annual harvested area of lodgepole pine in Alberta is approximately 20,000 ha with annual planting of about 8,000,000 trees and a projected annual planting of 15,000,000 trees by the turn of the century (Yang *et al.* 1997). With its important commercial status, lodgepole pine is a target species for intensive management.

1.1.2.3 The incidence of western gall rust in lodgepole pine

Western gall rust is one of the major fungal diseases in lodgepole pine. During the last several decades, high incidences of WGR in lodgepole pine have been reported frequently, especially in young plantations (Powell and Hiratsuka 1973; van der Kamp and Spence 1987; Bella and Navratil 1988; Yanchuk *et al.* 1988; Wu *et al.* 1996). A disease survey in a 6- to 12-year-old lodgepole pine plantation in Alberta showed that 63% of the sampled trees were infected by WGR (Powell and Hiratsuka 1973). Bella and Navratil (1988) reported a mortality of 30% caused by WGR in a 22-year-old lodgepole pine plantation in Alberta. In British Columbia, a study of a 21-year-old lodgepole pine provenance trial indicated that 66.8% of trees on average were attacked by WGR with a high incidence of up to 100% in one provenance (Wu *et al.* 1996). Greenhouse screenings also observed a substantial number of seedlings infected by WGR in lodgepole pine (Allen and Hiratsuka 1985; Blenis and Pinnell 1988; Yang *et al.* 1997). Due to the high incidence of WGR in some plantations and its potential threat to lodgepole pine, controlling the disease is becoming a primary concern in lodgepole pine management.

1.1.3 Traditional methods in control of western gall rust in hard pines

1.1.3.1 Removal of infected trees and branches

Traditionally, there are several methods to control WGR in lodgepole pine and other hard pine species. Removal of the infected trees and branches was often the first consideration in some areas because they are the sources of new infection. Moreover, WGR can infect from pine to pine directly, thus the rust disease cannot be controlled by the removal of alternative hosts. Rust incidence, however, can be reduced by the elimination of infected trees in a stand and in a protective zone around it (Ziller 1974). In intensive management situations, the eradication of the galls can be accomplished during the thinning operations (Hiratsuka 1987). Removal of the infected trees in and around the nursery should also minimize chances of infection for young trees. For growing healthy seedlings, the elimination of trees with the rust galls for a distance of 300 yards or more was recommended (Boyce 1961). In more heavily infected stands, however, the thinning or spacing operation may be difficult since WGR are commonly found on the larger trees as well (van der Kamp and Hawksworth 1985).

1.1.3.2 Chemical control methods

Timely protective fungicide application has also been used in controlling WGR disease. This method is effective and economically feasible in tree nurseries or in highly managed tree farms where the high-value ornamental pines are grown (Hiratsuka 1987). Merrill and Kistler (1976) reported that a single application of Maneb at the beginning of spore release greatly reduced the incidence of WGR in Scots pine (*Pinus sylvestris* L.) Christmas trees. Some other fungicides were also used to control WGR disease (Cunningham and Pickard 1985; Blenis *et al.* 1988). Blenis *et al.* (1996) showed that

Maltol treatment in lodgepole pine seedlings reduced the average percentage infection from 91.7% to 66.2%. However, the results of such chemical control are often inconclusive (Leaphart 1963), economically unfeasible and environmentally unacceptable in most forestry situations (Hiratsuka 1991b).

1.1.3.3 Biological control methods

Biological control has been considered as one of the favorable strategies for controlling the rust disease. The main objective of biological control is to use mycoparasites to control the epidemiology of the disease. Byler and Cobb (1969) reported several non-rust fungi associated with the *E. harknessii*. Some of these, such as species of the *Penicillium* and *Cladosporium* destroyed the aeciospores while others (*Gibberella lateritium*, *Doplodia pini* and *Nectria* sp.) invaded the living phloem and the xylem tissues of the gall. Such parasites contributed to the natural biological control of the disease. Several other mycoparasites, fungi and bacteria that associate with WGR have also been identified and their possible roles in controlling the pine rust disease have been investigated (Bergdahl and French 1978; Byler *et al.* 1972a, 1972b; Hiratsuka *et al.* 1979, Pickard *et al.* 1983; Tsuneda and Hiratsuka 1979; Tsuneda *et al.* 1980).

One of the important concerns in biological control of the rust is the deliverance of the mycoparasites among the galls. Insects and other free-moving organisms have been examined for their abilities to deliver the mycoparasites as the vectors and feed on the spores. For parasitizing WGR fungi, *Scytalidium uredinicola* Kuhlman, Carmichael & Miller is considered the best candidate mycoparasite and the beetle called *Epuraea obliquus* is chosen as the best vector for delivering the mycoparasite (Hiratsuka 1991b;

Chakravarty and Hiratsuka 1995; Currie 1995; Currie *et al.* 1995; Currie and Hiratsuka 1996). However, biological control of the rust disease is still under test, and the long-term effect of introducing mycoparasites and their delivering vectors into the forest ecosystem is still unknown.

1.1.3.4 Screening resistance to the disease

The resistance of hard pines to WGR is commonly observed. As in other hard pines, lodgepole pine exhibits a remarkable variation in the resistance to WGR. The selection for resistant trees could be made at the provenance, family and individual levels (Yanchuk *et al.*1988). Selection and breeding of the resistant stocks in lodgepole pine has always been one major strategy to control WGR disease during the past decade. In western Canada, the breeding for WGR resistance has become an integral part of the provincial lodgepole pine improvement programs (Dhir and Barnhardt 1993; Dhir *et al.* 1993). A conventional method for selection and breeding resistance to WGR usually starts with the evaluation of resistance in natural stands or in the provenance/family tests. Resistant provenances, families or individuals are then selected. However, such selection may not be effective, as the pathogenicity of the different WGR isolates has usually been ignored. Although a greenhouse screening can evaluate resistance more intensively and make the selection of resistant trees more efficient, the traditional method of selection and breeding for resistance to the rust will eventually be impeded without an understanding of the genetic mechanisms in host resistance.

1.1.4 Manipulation of both the rust and the host

1.1.4.1 General concept of manipulation of both the rust and the host

In the past, plant pathologists tried to control plant diseases by eradicating the disease pathogens. The aim of the approaches usually was to destroy the pathogens. However, diseases that could be controlled are few (Chaube and Singh 1991). As in other plant disease controls, the traditional methods to controlling WGR focused either on the rust (Merrill and Kistler 1976; Cunningham and Pickard 1985; Blenis *et al.* 1988) or on the hosts (Martinsson 1980; Yanchuk *et al.*1988; Klein *et al.* 1991). Such strategies may not be effective in controlling WGR disease in hard pines considering the host and the fungus are two dependent components of the forest ecosystem. Thus, an alternative strategy to manipulate both the rust and the host must be considered as our future management goal of lodgepole pine plantations. The goal of manipulation is not to eliminate the fungus from the hosts, but rather maintain the pathogen at an acceptable level in the hosts. By effective manipulation of both the host and the disease, the epidemic of the disease should be minimal.

1.1.4.2 Strategies and prerequisites toward the effective manipulation of both the rust and the host

As an obligate parasite, the dynamic of WGR depends on the availability of hosts it can parasitize. Virulent pathogens tend to colonize more hosts while the resistant hosts retard the increase of the pathogen population. The interaction between the host and the rust dominates their long-term co-evolutionary process and shapes the genetic structures of both the rust and the host. It is believed that such interaction between the rust and the host is governed by the resistance genes in the host and the virulence genes in the rust

(Flor 1971). Thus, an effective manipulation of both the rust and the host can be achieved eventually by monitoring the resistant stocks in the host according to the virulence level of the rust. Toward this goal, characterizing the virulence of varieties in the pathogen populations, isolating the resistance genes in the host and understanding the rust-host interaction are the primary requisites for the effective manipulation of both the rust and the host.

1.2 LITERATURE REVIEW AND DISCUSSION

During the past several decades, genetic studies in the host-pathogen pathosystem have been one of the most active research areas in plants, especially in agricultural crop species (Flor 1942, 1955, 1971; van der Plank 1963, 1968; Robinson 1976; Parlevliet and Zadoks 1977; Carson 1987). Early efforts focused mainly on the examination of host-pathogen interaction (Flor 1942, 1955, 1971; van der Plank 1963, 1968; Carson 1987). Later, with the development of genetic markers, quantification and characterization of virulence races in the pathogen using these markers became routine (Tuskan *et al.* 1991; Doudrick *et al.* 1993; Hamelin *et al.* 1994). Many molecular markers are available now and they are are widely used in genetic studies (Staub *et al.* 1996). The most striking progress by using the molecular markers in host-pathogen pathosystem research is the identification and localization of resistance genes in hosts. This part of the chapter will review the genetic studies in host-pathogen pathosystem in general and followed by the western gall rust-hard pine pathosystem in specific.

1.2.1 Genetic studies in host-pathogen pathosystem

1.2.1.1 General terms in describing host-pathogen interaction

Many hypotheses have been proposed to describe the genetics of host-pathogen systems, including the " gene-for-gene" system postulated by Flor (1942, 1955, 1971) in his pioneering genetic studies with the flax and flax rust *Melampsora lini* Ehrenb. Based on the gene-for-gene hypothesis, for each gene that conditions a resistance in the host there is a corresponding gene that conditions the pathogenicity in the parasite (Flor, 1971). Any resistance allele in the host acts if and only if there is an allele for the avirulence on a corresponding locus in the pathogen. This gene-for-gene interaction has been widely observed between the plants and the pathogens, including the fungi, viruses and bacteria. It has implicitly or explicitly served as the guiding principle in the breeding of crops for resistance to particularly fungal pathogens for most of this century (Thompson and Burdon 1992).

The other key issue to understanding the genetics of host-pathogen relation is the pattern of host resistance to the pathogens. van der Plank (1963, 1968) postulated that all disease resistance in plants could be classified into one of two categories; vertical and horizontal resistance. Vertical resistance operates against some races of the pathogen and not others while horizontal resistance operates equally against all races of the pathogen.

Vertical resistance has also been called differential resistance, field resistance, field immunity, hypersensitive resistance, major gene resistance, qualitative resistance, R-gene resistance, race-specific resistance, racial resistance, specific resistance (Robinson 1976). Horizontal resistance has been described as the general resistance, generalized resistance, multigenic resistance, non-hypersensitive resistance, minor gene resistance, quantitative resistance, polygenic resistance, race-non-specific resistance, non-racial resistance, non-

specific resistance, partial resistance, relative resistance and uniform resistance (Robinson 1976). However, these terms usually express their specific meanings in the context and consequently are not as commonly used as the vertical resistance and horizontal resistance.

It is generally assumed that vertical resistance is always inherited oligogenically and that polygenically inherited resistance can only be horizontal. However, not all horizontal resistance is polygenic and not all oligogenic resistance is vertical (Robinson 1976).

Vertical or horizontal resistance generally describes the resistance pattern in the hosts while the gene-for-gene concept focuses on modeling how the resistance genes in the hosts interact with the virulence genes in the pathogens. It has been proposed that both vertical and horizontal resistance operate against the pathogens on the gene-for-gene bases. All resistance genes in the host populations, either the genes with major effect or the genes with minor effect, interact in a gene-for-gene way with the virulence genes (Parlevliet and Zadoks 1977).

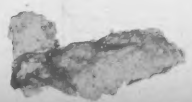
1.2.1.2 Molecular markers in genetic study of host-pathogen pathosystem

Characterizing the virulence variability in the pathogens and the localization of the resistance genes against the pathogens have always been the major focuses in genetic research of the host-pathogen pathosystem, given the fact of its great importance in understanding the nature of the host-pathogen relationship. However, great achievements in this research area have been made only recently as result of the development of molecular markers and associated technologies (Martin *et al.* 1991; Michelmore *et*

*al.*1991; Tuskan *et al.* 1991; Doudrick *et al.* 1993; Miklas *et al.* 1993; Hamelin *et al.* 1994).

Molecular markers provide an opportunity to study the genetic constitutions of the hosts and the pathogens at the DNA level. Genetic variability of the hosts and the pathogens can be quantified more accurately using the molecular markers (Tuskan *et al.* 1991; Doudrick *et al.* 1993; Hamelin *et al.* 1994). Molecular markers that are tightly linked to the resistance can be identified and utilized in the selection and the breeding for resistance to the pathogens (Martin *et al.* 1991; Michelmore *et al.*1991; Miklas *et al.* 1993). Genes responsible for resistance to the pathogens can be isolated with the help of molecular markers and other DNA manipulation technologies such as gene cloning and sequencing.

Molecular markers, which have been widely used in genetic studies, include protein markers such as isozymes and DNA markers such as RFLPs (restriction fragment length polymorphisms), RAPDs (random amplified polymorphic DNAs), SCARs (sequenced characterized amplified regions), ASAPs (allele-specific associated primers), SPARs (single primer amplification reactions), AFLPs (amplified fragment length polymorphisms), AP-PCR (arbitrary-primed polymerase chain reaction), SSCP (single-strand conformational polymorphism), CAPs (cleaved amplified polymorphic sequences), SSRs (simple sequence repeats) and STRs (short tandem repeats). The advantages and disadvantages of each molecular marker system were reviewed by Staub *et al.* (1996). Isozyme markers can provide genetic information as codominant markers. However, the paucity of isozyme loci and the fact that they are subject to post-translational modification often restricts their utility.



DNA markers such as RFLPs, RAPDs, STR, SSR, AFLPs etc. can be generated based on the entire genome of the organism. Thus they hold promise to provide an unlimited number of genetic markers. During the last several years, RAPDs analysis has been commonly used in many genetic studies because of its rapidity, simplicity and the need for a small quantity of genomic DNA (Chalmers *et al.* 1992; Dawson *et al.* 1995; Devey *et al.* 1995; Hubbes and Lin 1995; Sun *et al.* 1995. Yeh *et al.* 1995; Hua 1996)

1.2.1.3 Development of bulk segregation analysis (BSA) and its application in identification of molecular markers linked to resistance genes in host-pathogen system

The importance of identification of those molecular markers linked to the resistance genes stems from the fact that the genetic markers linked to the resistance genes could accelerate the progress in developing and deploying the resistant host stocks against the pathogens by marker-aided selection (MAS). Moreover, genetic linkage between the resistance genes and the molecular markers can be used to locate the resistance genes of the host using linkage maps, *in situ* hybridization and chromosome walking (Young 1990). Bulk segregation analysis is one of the methods used in detecting linkage between the molecular markers and the resistance genes in the host-pathogen pathosystem.

Bulk segregation analysis (BSA) was originally proposed by Arnheim *et al.* (1985) and later adopted by Michelmore *et al.* (1991). The method involves a comparison of two pooled DNA samples of individuals from a segregating population originating from a single cross. The DNAs from individuals showing the same phenotypic performance in trait of interest are pooled and analyzed. Molecular markers that are polymorphic between

the pools are identified and assumed to be the markers linked to the genes controlling the trait of interest.

Bulk segregation analysis has been widely used in the identification of linkage between RAPD markers and the disease resistance genes in the host-pathogen pathosystem. Michelmore *et al.* (1991) identified three RAPD markers linked to a gene resistant to the downy mildew (*Bremia lactucae*) in lettuce (*Lactuca sativa* cv. Safier × *L. serriola* PIVT1309) using BSA. In the common bean (*Phaseolus vulgaris*), a RAPD marker was quickly identified to be linked to a resistance gene to the common bean rust (*Uromyces appendiculatus*) (Miklas *et al.* 1993). The linkage between RAPD markers and the fusiform rust (*Cronartium quercuum* (Berk) Miyabe ex. Shirai f. sp. *fusiforme*) resistance genes in loblolly pine (*Pinus taeda* L.) was also detected by BSA (Wilcox, 1995). In sugar pine (*Pinus lambertiana* Dougl.), ten RAPD markers were found linked with the gene for resistance to white pine blister rust (*Cronartium ribicola*) using BSA (Devey *et al.* 1995).

BSA provides a rapid way to detect those molecular markers linked to the genes of interest. However, this method might be successful in only tagging genes of very large effect (Wang and Paterson 1994; Grattapaglia *et al.* 1996). For a quantitative trait in which many genes are involved and each gene has minor effect, it might not be possible to identify the linkage between molecular markers and the gene by BSA itself.

1.2.1.4 Development of QTL analysis and its application in identification of molecular markers linked to the resistance genes in host-pathogen pathosystem

Quantitative trait loci (QTL) analysis was made traditionally by relating the quantitative trait of interest in a group of individuals with their corresponding genetic markers. The genetic markers that are significantly associated with the trait of interest are declared a linkage with the QTL. Since the association between the genetic markers and the traits of interest is analyzed statistically, the QTL analysis has the potential to detect the linkage between the genetic markers with the genes of interest and quantify their genetic effects.

Early attempts to detect quantitative trait loci controlling commercially important traits were made mostly in crop species. The identification of quantitative trait loci was simply carried out by relating the quantitative traits of interests with a few of the morphological markers (Sax 1923; Thoday 1961; Law 1967). Later, as the enzyme markers became available, the detections of quantitative trait loci were performed by relating the quantitative traits with the enzyme markers (Tanksley *et al.* 1982; Edwards *et al.* 1987). These early genetic studies showed that it was possible occasionally to relate the genetic linkage to the putative quantitative trait loci. However, systematic and accurate mapping of QTLs had not been possible due to the difficulty in arranging crosses with genetic markers that were densely spaced throughout the entire genome (Lander and Botstein 1989).

Molecular genetic markers such as restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD) developed recently have made it possible to construct a relatively high density genetic map in many organisms. The advent of MAPMAKER-QTL computer program that implemented interval mapping methods facilitated the systematic search for QTL effects. The interval mapping approach allows

the detection of QTLs at any point flanked between the markers on the linkage map based on their recombination frequencies. Thus, it increases the power and accuracy of the QTL detection compared to traditional single marker one-at-a-time method (Lander and Botstein 1989). By first using a complete linkage map of restriction fragment length polymorphisms (RFLP) and interval mapping technology, Paterson *et al.* (1988) detected and mapped six QTLs controlling the fruit mass, four QTLs for the concentration of soluble solids and five QTLs for fruit pH in tomato (*Lycopersicon esculentum* cv. UC82B × *L. chemielewskii* LA1028). The QTLs identified for fruit mass, soluble solid, and pH accounted for 58%, 44% and 48%, respectively, of the total phenotypic variance. QTLs that control important quantitative traits were also mapped for many other crop species (Stuber *et al.*1992; Veldboom *et al.* 1994.).

In forest trees, the systematic identifications of QTLs for commercially important traits were conducted only recently. Groover *et al.* (1994) identified and mapped five QTLs influencing the wood specific gravity in an outbred pedigree of loblolly pine (*Pinus taeda* L.) based on its RFLP genetic map. The five QTLs explained 23% of the total phenotypic variance for wood specific gravity. In Populus (*Populus trichocarpa* × *P. deltoides*), five QTLs explained 84.7% of the genetic variance in spring flush date and another two QTLs accounted for 44.7% of the genetic variance in two-year stem volume growth (Bradshaw and Stettler 1995). QTLs that control vegetative propagation traits in Eucalyptus (*Eucalyptus grandis* × *E. urophylla*) were also mapped based on its RAPD genomic map (Grattapaglia *et al.* 1995). In Cocoa (*Theobroma cacao* L.), four QTLs for early flowering, trunk diameter, jorquette height and ovule number have been identified (Crouzillat *et al.*, 1996).

QTLs that confer disease resistance have also been identified and mapped in crops. In maize (*Zea mays* L.), five QTLs that control resistance to stalk rot (*Gibberella zeae* (Schw.) Petch) were detected and mapped onto the chromosomes (Pé *et al.* 1993). Four QTLs that confer resistance to common blight (*Xanthomonas campestris* pv. *phaseoli* (Smith) Dye) were identified in the common bean (*Phaseolus vulgaris* L.) (Nodari *et al.* 1993). In barley (*Hordeum vulgare*), two QTLs that confer resistance to the barley stripe rust (*Puccinia striformis* f. sp. *hordei*) were mapped (Chen *et al.* 1994). One of the two QTLs had a major effect, accounting for 57% of the variation in disease severity. The other QTL had a minor effect, controlling 10% of the total phenotypic variance.

The identification of QTLs that control disease resistance in forest trees was conducted only recently. Newcombe and Bradshaw (1996) detected two QTLs that confer resistance to the leaf spot disease (*Septoria populicola* Peck) in the hybrid popar (*Populus trichocarpa* × *P. Deltoides*). The two QTLs each explained 44.8% and 36.9% of the total phenotypic variance in disease resistance in the 4th-year growth of the hybrid popar.

The systemic identification and mapping of QTLs will require a genetic linkage map densely spaced with genetic markers. The successful construction of a genetic linkage map relies on the abundance of the segregating markers and the estimation of the recombination frequency between them. In crops, F_1, F_2, backcross, inbred line and doubled haploid populations are commonly used for the map construction (Staub *et al.* 1996).

In forest trees, QTL mapping was carried out using three-generation pedigrees, two-generation pedigrees and open-pollinated populations. The construction of the three-generation outbred pedigrees involved crosses between the grandparental pairs and the

parental pairs such as in loblolly pine (Groover *et al.* 1994) or involved backcross between F1 and one of its parents, such as in Cocoa (*Theobroma cacao* L) (Crouzillat *et al.* 1996). In *Populus*, the three-generation mapping pedigree included interspecific hybridization and its F1 and F2 progenies (Bradshaw and Stettler 1995). The construction of the two-generation mapping pedigree usually involves crosses between the highly heterozygous parents. Such pseudo-testcross mapping strategy has been successfully used in the *Eucalytus* (Grattapaglia *et al.* 1996).

Given the fact of relatively long generation intervals in forest trees, the construction of either a three-generation pedigree or a two-generation pedigree is time and labor intensive. Thus, an alternative mapping strategy using open-pollinated families has been investigated in *Eucalyptus grandis* and QTLs that control growth and wood quality have been detected (Grattapaglia *et al.* 1996). However, the use of open-pollinated families in *Eucalypus grandis* in QTL mapping was not straightforward. The genetic markers had to be selected to be present and heterozygous in the maternal tree, and absent, homozygous null in the pollinators (Grattapaglia *et al.* 1996).

The special haploid megagametophyte feature in conifers makes it possible to use open-pollinated families directly in the map construction and the QTL identification. In conifers, heterozygous loci in the maternal tree will segregate in a 1:1 ratio in its haploid megaspores. A single haploid megaspore of the maternal tree contributes to the development of the both haploid megagametopyte and the embryo. Thus, half of the genetic materials in the embryo are identical to that of the corresponding megagametophyte. Consequently, haploid megagametophytes of an open-pollinated family harvested from the germinating seedlings can be used to construct the linkage map

by co-segregation analysis of the genetic markers. The corresponding seedlings can be used for evaluation of the quantitative traits. The QTL mapping can be carried out by relating the genetic markers in the linkage map with the corresponding quantitative traits. However, the identification of QTLs based on genetic linkage maps in lodgepole pine using such open-pollinated family approach has not been reported to date.

1.2.2 Genetic studies in the western gall rust and lodgepole pine pathosystem

1.2.2.1 Genetic variability in western gall rust fungus

Western gall rust fungus is an endocyclic, autoecious rust, possessing only one stage of spores. The fungus has a broad geographic range, from the Unites States to Canada, and a wide host species, infecting about 20 native and exotic hard pines. Early studies on the fungus mainly focused on the taxonomic classification and the biology (Anderson and French 1965; Hiratsuka *et al.*1966; Hiratsuka and Maruyama 1968; Hiratsuka 1969). Genetic variation in the pathogen populations has been explored only during the last ten years. Tuskan *et al.* (1991) examined the variability of 201 WGR fungal isolates using starch gel electrophoresis. The 201 isolates were collected from 13 distinct geographic locations throughout North Dakota and northwest Minnesota. The study involved three host species, the ponderosa, jack and Scots pines. Five of the 13 putative loci were found polymorphic and their frequencies were heterogeneous among the locations. The Fst apportioned 51.3% and 3.9% of the total isozyme variation to the differences among the locations and among the host species, respectively. The small isozyme difference among isolates from different *Pinus* species might suggest that selective pressure for the host specificity in WGR fungus in sampled populations was minimal.

The lack of host specificity in WGR fungus was also apparent in a study of isozyme variability among 341 isolates collected from 13 *Pinus* species at 39 locations from the Pacific Coast and Cascade-Sierra Nevada in California, and five locations from Idaho, Montana and the Cascade-Sierra Nevada in Oregon (Vogler *et al.* 1991). Nine of the 15 putative loci were monomorphic. The six remaining polymorphic loci separated the isolates into two groups. Within each, all isolates exhibited an identical electrophoretic profile. One group with 252 isolates originated from all 13 host species. The second group was composed of 89 isolates from three host species. The greater isozyme differentiation among the isolates in Tuskan *et al.* (1990) were attributed to possible inclusion of both the western and eastern spore sources (Tuskan *et al.* 1990; Vogler *et al.* 1991).

Recently, molecular techniques have enabled the study of geographic variability in WGR fungus at the DNA level. Analysis of restriction fragment length polymorphisms in 25 single-gall aeciospores from lodgepole pine host at five locations in British Columbia revealed variability within and among locations at the ribosomal DNA region (Sun *et al.* 1995). A study of the random amplified polymorphic DNA (RAPD) in the WGR fungal isolates from lodgepole pine host at 12 locations across British Columbia also showed variability within and among the locations (Sun *et al.* 1995). In Ontario, the WGR fungal isolates sampled from jack pine hosts exhibited variability at 16 of 24 RAPDs, but the geographic variability among the isolates was not apparent, probably due to limited sample size (Hubbes and Lin 1995). Hence, in contrast to the extensive sampling of WGR fungal isolates in isozyme studies in the USA (Tuskan *et al.* 1990; Volger *et al.* 1991), the molecular studies of the WGR fungus in Canada each aimed at a restricted

distribution of the rust. Consequently, the broader pattern of geographic variability of the WGR fungus in Canada is unknown, in particular, when different host species are involved.

1.2.2.2 Resistance variation in lodgepole pine

Lodgepole pine is one of the major hosts of the WGR fungus and one of the primary reforestation species in North America. Knowledge of the variability in resistance to the WGR will help develop a sound strategy in selecting and breeding the resistant stocks in lodgepole pine. The evaluation of resistance performances in lodgepole pine was usually carried out by a field investigation of different provenances and different families grown and exposed to natural sources of the rust. At the beginning of the 1970s, a series of lodgepole pine provenance trials were established by the British Columbia Forest Service in several locations in B.C. and the Yukon Territory. Field observations in these provenance trials showed that lodgepole pine demonstrated remarkable variation in the resistance to WGR disease. In the 5-year- old provenance trial of 53 lodgepole pine provenances located at Red Rock, Martinsson (1980) observed that the high elevation and the high latitude lodgepole pine provenances were particularly susceptible to the WGR infection. Yanchuk *et al.* (1988) reported a 10-year-old progeny test near Red Rock B.C. which contained 214 open-pollinated lodgepole pine families from 24 provenances. The variation in resistance to the WGR disease in lodgepole pine existed at the level of provenance, population within provenance and the individual, with the coastal provenances showing the highest infection score. Wu *et al.* (1996) investigated the incidence of the WGR disease in a 21-year old provenance-family test plantation in Red

Rock, B.C. The plantation contained 778 different wind-pollinated families from 53 provenances from the western Canada. The results indicated that the resistant provenances tended to be concentrated in the northeast part of the lodgepole pine's natural distribution such as the Peace River region and along the low elevation sites of the wetbelt of southern interior B.C. The most susceptible provenance had an infection rate of 100% while the resistant provenance had only 17% of the infection incidence.

Variation of resistance in lodgepole pine to the WGR disease was also revealed in a greenhouse screening by artificial inoculation. Yang *et al.* (1997) inoculated 291 open-pollinated families originating from west central Alberta with a mixture of WGR spores collected from Hinton, Alberta. They observed an east-west trend of resistance performances, with the western and the high-elevation families being more susceptible to the WGR infection. The geographic trends of the WGR susceptibility in lodgepole pine detected by Yang *et al.* (1997) are comparable to some extent to the field observations reviewed above, i.e. the provenances and families from the low elevation and from the contact regions with jack pine (*Pinus banksiana* Lamb) are more resistant to the WGR.

It seems that resistance to WGR in lodgepole pine is controlled by many resistance factors. It was found that resistance varied in a continuous fashion when expressed as the number of infections per tree (van der Kamp 1989). In greenhouse studies, seedlings with no galls, complete galls as well as partial galls were commonly observed among the individuals of lodgepole pine (Yang *et al.* 1997). Kojwang and van der Kamp (1991) proposed that a polygenic resistance in lodgepole pine to WGR predominates but single major resistance may also play a role in lodgepole pine. It has also been suggested that the resistance genes in lodgepole pine appear to interact multiplicatively (van der Kamp and

Tait 1990; van der Kamp 1991). However, more evidence is still needed in order to make a final conclusion regarding the genetic base of the resistance to WGR in lodgepole pine.

1.2.2.3 Western gall rust and lodgepole pine interaction

An understanding of the host-parasite interaction in the lodgepole pine-western gall rust pathosystem will provide fundamental information for effective manipulation of both hosts and disease. During the last decades, several studies have investigated resistance features in the lodgepole pine-western gall rust pathosystem. These studies usually tested the interaction between lodgepole pine and WGR by inoculating the different provenances, the families, or even the clones of lodgepole pine with different sources of the WGR spores. van der Kamp (1988b) inoculated seven lodgepole pine provenances with four WGR spore sources and detected significant interaction between the provenances and the spore sources. Two coastal provenances were significantly more resistant to the three interior spore sources than to the coastal spore sources, whereas the other provenances did not vary much in their resistance to the four spore sources. A similar inoculation study involving 28 lodgepole pine and Scots pine seed lots was conducted by van der Kamp (1989) using two different WGR spore sources, one from Scots pine host, and another from the lodgepole pine host. The two rust collections did not show a significant difference in their virulence spectrum and there was no significant interaction between the spore source and the pine seed lots.

In Alberta, the interaction between the lodgepole pine and the WGR was examined by Blenis *et al.* (1993). Twenty-three open-pollinated families from five stands in west central Alberta were inoculated with four WGR spore sources that were collected within

60 km of the host stands. There was no significant interaction between WGR spore sources with the stands and the families.

According to van der Plank (1968) vertical resistance implies a differential interaction between the varieties of the host and races of the pathogen. In horizontal resistance there is no differential interaction. Thus, resistance can be analyzed by simple statistical methods. However, selection of host and pathogen differentials is important to successfully test such interactions.

In the western gall rust and pine pathosystem, the test of interaction between the host and the rust may provide an insight into the key issues of vertical and horizontal resistance. However, these issues still remain unclear. Previous studies to test lodgepole pine and western gall rust interaction resulted in different conclusions. Significant interaction and non-significant interaction between the WGR sources and the lodgepole pine were both observed (van der Kamp 1988b; van der Kamp 1989; Blenis *et al.* 1993).

The traditional methodology used in lodgepole pine and WGR interaction studies might account for the discrepancies of the results. First, the mixed spore sources were usually produced by collecting spores from different galls and then mixing together. Thus, it is difficult to refer the different spore sources as pathogen differentials without knowing their genetic backgrounds. Consequently the interaction between the rust and the host could be confounded by the mixture of spore sources.

The criterion used to evaluate the resistance to WGR in lodgepole pine may also affect the outcome in lodgepole pine and WGR interaction analysis. So far several parameters have been used to measure the severity of infection by WGR in lodgepole pine. Frequency or incidence of infection and area under the disease progress curve

(AUDPC) were used to measure the severity of infection at population level (Martinsson 1980; van der Kamp 1988a; van der Kamp 1989; Blenis *et al.* 1993; Wu *et al.* 1996). The measurements were based on the count of infected individuals and non-infected individuals. The variations of the reaction to the rust among the infected individuals, however, were ignored.

At the individual level, the number of galls per tree or the number of infections per tree was usually used to measure the infection severity of a single tree. They are either recorded as actual number of galls or infections per tree (van de Kamp and Tait 1990; Kojwang and van der Kamp 1991) or as categories, each representing a range of galls per tree (Yanchuk *et al.* 1988, Wu *et al.* 1996). However, this criterion may not be very effective in the evaluation of resistance in those seedlings in which only part of their crowns was exposed to the inoculum.

The 0-5 rating scales of infection, used by Klein *et al.* (1991) to describe the response of jack pine to the infection of western gall rust, was also used to measure the infection level of lodgepole pine to WGR at the individual level (Blenis *et al.* 1993, Yang *et al.* 1997). The 0-5 rating scale focuses on the gall morphology of each single gall, with a 0 representing no infection, and a 5 representing a complete gall. Any partial galls are classified between 1-4 (Klein *et al.* 1991). This criterion measures the disease reactions for each individual and is suitable for artificial inoculation using the torn needle method (Myrholm and Hiratsuka 1993).

Some of the interaction between lodgepole pine and WGR was tested at the provenance level. Since the provenance may consist of individuals with great variability in their genetic constitutions, the variation in resistance among the individuals may be

confounded with the provenance effect, making it more difficult for the interaction to be detected.

Recently, the interaction between WGR spore sources was investigated at the clone level. Kojwang and van der Kamp (1991) developed sixteen clones of lodgepole pine by grafting, taking advantage of the genetic uniformity of clones. These sixteen clones had a physiological age of 22 to 25 years and were inoculated with four WGR spore sources. Each of the four spore sources came from a single gall of WGR. Significant clone effect, inoculum effect and interaction between the clones and the rust sources were detected. The disease severity was evaluated as number of galls per tree. The result lead to the conclusion that the lodgepole pine and the western gall rust pathosystem appears to be a mixed system in which both horizontal and vertical effects occur with horizontal effects predominating (Kojwang and van der Kamp 1991).

Considering WGR has an extensive distribution infecting more than 20 native and exotic pine species, great virulence variability in the rust should be expected. Undoubtedly, the efficiency of either screening resistance to the rust or test of interaction between the rust and the host will be increased by using genetically different inoculums and a proper resistance evaluation system.

1.2.2.4 The identification of genetic markers linked to resistance to western gall rust in lodgepole and other hard pines

In the past several years, great progress has been made in the identification of genetic markers linked to disease resistance genes in plant species. Unfortunately, such research in pine has been limited to the study of resistance to WGR. Only recently, Hua (1996)

identified a candidate RAPD marker linked to resistance in jack pine. To understand the genetic basis of resistance to WGR and to effectively manipulate both the rust and the host, there is a need to study the genetic basis of the pathosystem between the rust and the host.

1.3 STUDY OBJECTIVES AND GENERAL EXPERIMENTAL DESIGN

1.3.1 Study objectives

During the past several decades, studies of WGR and its hosts provided the fundamental information towards understanding the host, the pathogen and the host-pathogen relationship. However, little progress has been made in the description and the isolation of the mechanisms and the genetic components that determine the outcome of the host-pathogen interaction. Moreover, the magnitude of variability in WGR fungus remains unclear, especially when broader distribution and different hosts are considered. The examination of interaction between the rust and the host using genetically different isolates is still necessary. The objectives of this study therefore were:

(1). Characterizing and quantification of genetic variability in WGR populations collected from different hosts and locations using molecular markers.

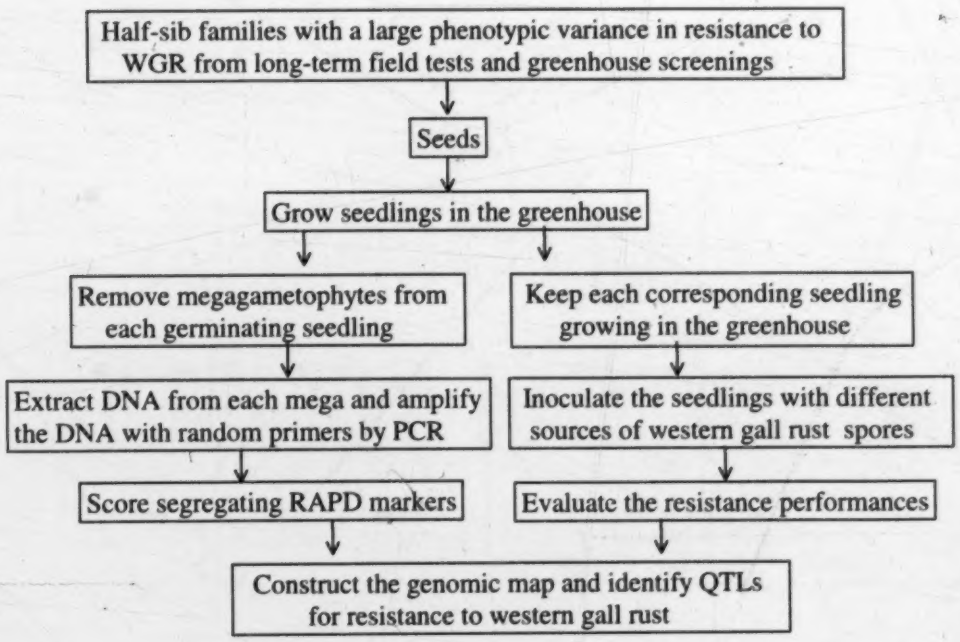
(2). Evaluation of resistance performances in different lodgepole pine families inoculated with genetically different WGR isolates.

(3). Construction of high density genetic maps in lodgepole pine using molecular markers to facilitate genetic studies and quantitative trait (QTL) analyses in lodgepole pine.

(4). Identification and mapping quantitative trait loci for resistance to WGR in different lodgepole pine families based on their genetic maps.

1.3.2 General experimental design

This study involves two parts. The first part is to quantify the variability of WGR using RAPD markers. The second part, which is depicted in the following diagram, is to construct genetic linkage maps and identify QTLs conferring resistance to different WGR sources in lodgepole pine.


Half-sib families with a large phenotypic variance in resistance to WGR from long-term field tests and greenhouse screenings
Seeds
Grow seedlings in the greenhouse
Remove megagametophytes from each germinating seedling
Keep each corresponding seedling growing in the greenhouse
Extract DNA from each mega and amplify the DNA with random primers by PCR
Inoculate the seedlings with different sources of western gall rust spores
Score segregating RAPD markers
Evaluate the resistance performances
Construct the genomic map and identify QTLs for resistance to western gall rust

To investigate genetic variation of WGR more thoroughly, we will sample single gall spores across Canada from both jack pine and lodgepole pine hosts and quantify the variability of the rust populations using RAPD markers. Variation patterns in WGR will

be identified and the predominant isolates of the rust will be used in resistance evaluation and QTL analyses in lodgepole pine.

Construction of the genetic map and identification of QTLs for resistance will be conducted using half-sib families with a large phenotype variance in resistance to WGR. In conifers, half-sib families are a desirable alternative for map construction when full-sib families are not available. The haploid megagametophytes are used for map construction, while the embryo of the seed, which contains the genetic material of the corresponding megagametophyte, will be grown and used for evaluation of resistance. Identification of genetic markers for resistance is possible by relating genetic markers to resistance performances. Half-sib families with a larger phenotypic variance in resistance will increase the possibility in identification of genetic markers for resistance since in such cases the resistance genes are likely to be segregating among the megagametophytes and the embryos. In this study, QTLs for resistance to WGR will be identified for three families against two different types of western gall rust sources.

The following chapters of the thesis will address the issues specified in this study. Chapter 2 will deal with the study of the genetic variability in WGR. Chapter 3 will focus on evaluation of resistance performance of the three lodgepole pine families inoculated with two different WGR isolates. Chapter 4 will report the construction of genetic maps in the three lodgepole pine families. Chapter 5 will summarize the identification of quantitative trait loci for resistance to WGR in the three lodgepole pine families. In Chapter 6, a general discussion and implication of the results will be addressed.

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CHAPTER 2 RANDOM AMPLIFIED POLYMORPHIC DNA VARIABILITY AMONG GEOGRAPHIC ISOLATES OF WESTERN GALL RUST FUNGUS

2.1 INTRODUCTION

The rust fungus *Endocronartium harknessii,* which causes western gall rust (WGR) in hard pines, is an endocyclic rust possessing only one spore state and has no alternative hosts. The fungus infects more than 20 native and exotic hard pine species and is found throughout western and northern North America (Tuskan and Walla 1989). In Canada, lodgepole pine (*Pinus contorta* var. *latifolia.*) and jack pine (*Pinus banksiana*) are two major hosts of WGR. The geographic range of lodgepole pine is confined to southwestern Alberta and much of British Columbia while that of jack pine extends from the Maritime provinces to northeastern Alberta (Hosie, 1979). Traditionally, efforts to control WGR fungal infection have focused on either containing the fungus (Tsuneda and Hiratsuka 1979; Blenis *et al.* 1988; 1996; Currie *et al.* 1995) or increasing genetic resistance in the host (Martinsson 1980; Yanchuk *et al.* 1988; Kojwang 1994). However, as the management of lodgepole pine plantations becomes intensive, the effective manipulation of both fungus and hosts must be considered the prime objective.

An understanding of the pattern of variability in WGR fungus and its host preference is a prerequisite for the effective management of this pathogen in lodgepole pine plantations. During the last decade, the variability of WGR was examined using isozyme or RAPD markers. Tuskan *et al.* (1990) investigated the isozyme variability of WGR isolates from 13 locations in North-Central United States and found most of the variation was attributed to the differences among the locations. The isozyme difference among

isolates from different host species was minimal. Vogler *et al.* (1991) detected two zymodemes of WGR isolates from 44 locations in the western United States. With each zymodeme all isolates exhibited identical electrophoretic profiles. The extensive studies on genetic variability of WGR in the United States are beneficial for understanding the potential virulence variation of the rust, and for selection of genetically divergent sources in screening resistance programs. In Canada however investigations of variability of WGR has been restricted to British Columbia (Sun *et al.* 1995) and Ontario (Hubbes and Lin 1995). Consequently, the broader pattern of geographic variability of WGR fungus in Canada remains unclear, particularly when different host species are included.

RAPDs have been shown to mostly inherit in a biparental dominant Mendelian manner (Carlson *et al.* 1991; Roy *et al.* 1992; Heun and Helentjaris 1993; Rieseberg *et al.* 1993) and their use as markers in population genetic study has been well established (Chalmers *et al.* 1992; Huff *et al.* 1993; Lynch and Milligan 1994; Dawson *et al.* 1995; Yeh *et al.* 1995). RAPDs are particularly attractive for studying variability in WGR fungus because only a small quantity of DNA is required for analysis.

The objective of this study was to provide baseline data on the pattern of variability in WGR fungus collected from lodgepole pine and jack pine across Canada using RAPD markers. The results are reported in this chapter.

2.2 MATERIALS AND METHODS

2.2.1 Collection of fungal spores

Seventy-three single galls were collected from lodgepole pine and jack pine hosts at 13 locations in May and June of 1994. They were kept separate by gall and brought back

to the laboratory in paper bags. The locations represent four lodgepole pine origins in British Columbia and Alberta and nine jack pine origins in Saskatchewan, Manitoba and Ontario (Table 2-1 and Figure 2-1). Only the inner layer of aeciospores was collected as a WGR fungal isolate of each gall by brushing and sieving through a thin nylon cloth. Cleaned aeciospores were kept separately by gall, air dried for 24 hr. and stored at -20^{0}C.

2.2.2 DNA extraction

Total DNA was extracted from spores for each isolate using a method modified from that described by Lee and Taylor (1990). Briefly, 20 mg of spores and 10 mg washed sea sand (Fisher Scientific) were mixed and grounded by hand with a small pestle in a 5 ml microcentrifuge tube. Fifty microlitres lysis buffer (50 mM Tris-HCl, pH 7.2, 50 mM EDTA, 3% SDS, 1% 2-mercaptoethanol) was added and the mixture was transferred to a 1.5 ml microcentrifuge tube and incubated at 65 ^{0}C for 1 hour. After incubation, 50 µl chloroform:phenol (Applied Biosystems Inc.) was added and vortexed briefly, then centrifuged for 10 min at 10,000 g. The upper phase was extracted with 50 µl chloroform:octanol (24:1, v:v). The supernatant was precipitated with a 10% final volume of 3 M sodium acetate (pH 5.2) and 2-2.5 volume of 90% cold ethanol. The precipitate was pelleted, rinsed with 70% ethanol, air dried at room temperature and resuspended in 50 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 100µg/ml of RNAse A (Boehringer Mannheim) was added and the solution was incubated for 1 hour at 37 ^{0}C. Concentration of DNA was adjusted to 1 ng/µl. The DNA samples were stored at -20 ^{0}C.

2.2.3 DNA amplification

Each amplification was carried out in a volume of 20 µl containing 50 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, 300 ng/ml BSA, 0.5% Ficoll, 1 mM Tartrazine, 200 µM each of dATP, dCTP, dGTP and dTTP, 0.20 µM random primer, 10 ng of WGR fungus DNA, and 1 unit *Taq* DNA polymerase (Boehringer Mannheim). Samples were loaded into micro-capillary tubes and sealed by flame using a laboratory burner. Amplification was performed in a 1605 Air Thermo-Cycler (Idaho Technology), programmed for 40 cycles of 1 min of denaturing at 91^0C, 1 min of annealing at 36^0C, and 2 min of extension at 72^0C after an initial denaturation of 2 min at 94^0C. The program was followed by an extension phase at 72^0C for 8 min. A sample without WGR fungal DNA was the negative control. Amplified RAPD products were directly loaded into a 1.4% agarose gel from the capillary tubes and electrophoresis was performed at 100 V for 2 hours. Gels were stained in ethidium bromide solution and photographed under illumination with UV-light.

Fifty UBC Set-3 (University of British Columbia Biotechnology Laboratory, Vancouver, B.C) and 40 Operon (Operon Technologies, Inc., Alamedia, Calif.) Set-A (OPA) and Set-B (OPB) random oligonucleotide primers were initially screened on four WGR fungal isolates sampled from four locations. Five UBC (UBC266, UBC283, UBC285, UBC289 and UBC290) and four Operon primers (OPA02, OPA07, OPA13 and OPA14) that consistently revealed sharp and reproducible RAPDs (fragments) over several independent runs were chosen for this study. The nine primers are each 10 nucleotide in length and have approximately 60% G/C ratio in content.

2.2.4 Data analysis

Photographs from ethidium bromide-stained agarose gels were used to score the data for RAPD analysis. We named each RAPD by the primer identity and a hyphenated numeral corresponding to the molecular size. Two phenotypes, marker and null, were detectable at each RAPD. Since RAPD markers are dominant, RAPD was scored as 1 if the marker was present, representing either homozygote of the marker or heterozygote of the marker/null phenotype. A RAPD was scored as 0 if the marker was absent, representing null homozygotes. Thus, each of the 73 WGR fungal isolates was coded by a vector of 1s and 0s, representing its RAPD multi-band phenotype or fingerprint.

The array of 1s and 0s at each variable RAPD was tabulated by location and by host. Contingency X^2 tests were used to determine the homogeneity between hosts and between locations within hosts. The pair-wise RAPD distances among the 73 WGR fungal isolates were estimated from the Euclidean distance of Excoffier *et al.* (1992), defined for RAPDs by Huff *et al.* (1993) as:

$$E_{ij} = m\,(1 - (2m_{ij} / m)) \qquad [1]$$

where m_{ij} was the number of RAPDs shared by two isolates i and j, and m was the total number of variable RAPDs. An analysis of molecular variance (Excoffier *et al.* 1992) of the resultant matrix of Euclidean distances partitioned the RAPD variability to the among-host, among-location within the hosts and within-location variance components. A non-parametric permutational procedure computed the significance of variance component (Excoffier *et al.* 1992).

Pair-wise RAPD distances between locations were computed from the following equation:

$$D_{(ij)} = \sum_{i=1}^{n_x} \sum_{j=1}^{n_y} (E_{ij(xy)} / n_x n_y) \qquad [2]$$

where $E_{ij(xy)}$ was the distance between isolate i from location x and isolate j from location y, as defined in equation [1], n_x and n_y were the number of isolates from location x and location y, respectively. Pair-wise comparisons of the 13 locations constituted a matrix of average distance between locations. This matrix was used in cluster analysis with an option of UPGMA (unweighed pair group method, arithmetic average) to construct a dendrogram that depicted the hierarchical structure of RAPD affinity among geographic isolates of WGR fungus.

2.3 RESULTS

The nine random primers chosen for analysis generated 41 RAPDs that ranged in size from 350 to 2500 bp. Each primer amplified between two to seven RAPDs. Examples of RAPD profiles from primers UBC290 and OPA07 are shown in Figure 2-2. Eighteen of the 41 RAPDs (43.9%) were polymorphic (presence of marker and null phenotypes) in the WGR fungal isolates sampled from lodgepole pine and jack pine hosts. Their occurrence (count of marker phenotype) by host and contingency X^2 tests of the heterogeneity of occurrence between the hosts are in Table 2-2.

Fifteen of the 18 polymorphic RAPDs (83.3%) could discriminate the WGR fungal isolates of lodgepole pine from jack pine host origins (Table 2-2). Of these 15 RAPDs, 10 (66.7%) provided marker phenotypes that were unique to the isolates of lodgepole or jack pine hosts. The remaining five RAPDs (34.3%) were significantly heterogeneous ($P \leq 0.01$) in distribution between the two hosts. Therefore, RAPD divergence of the WGR fungal isolates sampled from lodgepole pine and jack pine hosts in this study was mainly due to the presence and absence of the unique marker phenotypes rather than to difference in RAPD frequency.

The thirty western gall rust fungal isolates from lodgepole pine hosts at the four locations in British Columbia and Alberta exhibited an identical RAPD profile (results not shown). However, counts of marker phenotype at four (UBC289-1900, OPA02-950, OPA02-510, OPA07-970) of eight polymorphic RAPDs were significantly heterogeneous among the nine geographic origins in jack pine hosts (Table 2-3). Only three of the eight geographic origins (J4, J5, and J9) had segregating RAPDs, and in two cases a segregating rare RAPD was restricted to a single population (OPA07-970 in J9, OPA13-690 in J5). Of the six geographic origins (J1-J3 and J6-J8) without segregating RAPDs (monomorphic), three were each unique to a province.

Analysis of molecular variance (Table 2-4) revealed the among-host component accounted for 76.3% of the total RAPD variability and was significantly different from zero at a 0.001% level of probability. The components due to among locations within hosts and within locations accountrd for 14.4% and 9.3% respectively of the total RAPD variability, and were also significantly different from zero at the 0.001% level of probability. The dendrogram depicting RAPD affinity among the WGR fungal isolates

sampled at 13 locations revealed a major separation between lodgepole and jack pine hosts, and an east-west trend of decreasing similarity among the isolates sampled from jack pine hosts (Figure 2-3, Figure 2-4).

2.4 DISCUSSION

In this study of WGR fungus sampled from British Columbia to Ontario, the most significant finding was the great RAPD differentiation between isolates of lodgepole pine and jack pine host origins. The count of marker phenotype at 15 of 18 polymorphic RAPDs were significantly heterogeneous ($P \leq 0.01$) and of these, ten were host specific (Table 2-2). Analysis of molecular variance apportioned 76.3% of the total RAPD variability in WGR fungal isolates to differences between lodgepole and jack pine hosts. This high level of differentiation between isolates from lodgepole pine and jack pine hosts might be the result of the coevolutionary processes in associations with strong differential selection pressure against the WGR fungus imposed by these two hosts.

Host-specific RAPD differentiation in this study contrasts with the small isozyme difference among the isolates from three pine hosts in North Dakota, Nebraska and Minnesota (Tuskan *et al.* 1990) and from 13 pine hosts in the Pacific Coast, Cascade-Sierra Nevada in California and Oregon, Idaho and Montana (Vogler *et al.* 1991). This might illustrate an inherent difference among sampled populations in different studies and/or represent a greater resolution of RAPDs relative to isozyme markers in delineating WGR fungal isolates. The limitations of isozyme survey of variations were documented (Yeh 1989) and numerous isozyme studies failed to detect variation in rusts that showed

high levels of pathogenic variability (Newton *et al.* 1985; Leung and Williams 1986; McCain *et al.* 1992).

Host-specific RAPD differentiation was reported for other fungi. Tham *et al.* (1994) found that RAPD patterns in *Peronospora parasitica* (Pers.ex Fr.) were highly correlated with the hosts and that use of only two decamer primers could discriminate among the pathotypes in different hosts. Host specific RAPDs might reflect the designation of parasites to different hosts. Further tests of this hypothesis would be very informative for manipulation of WGR and the hosts.

RAPD variability within the hosts was detected only among the isolates sampled in jack pine (Table 2-3). Four of the eight RAPDs were significantly heterogeneous in occurrence among the locations and there was an east-west trend of decreasing relatedness (Figure 2-3, Figure2-4). This clinal pattern of differentiation might suggest an adaptation of the WGR fungus to local environments in jack pine plantations. However, non-selective historical events, such as past migration patterns, changes in population size and colonization, with associated founder effects (Endler 1977), could also influence the distribution of RAPDs in the WGR fungal isolates sampled from jack pine hosts. The greater variability in RAPD profiles among the isolates in Ontario and Manitoba is consistent with the preliminary result of a RAPD survey of WGR fungal isolates in Ontario (Hubbes and Lin 1995).

Uniformity of RAPD profile among the 30 WGR fungal isolates sampled by us from lodgepole pine hosts in British Columbia and Alberta contrasts with the large RAPD variability observed previously among the 96 WGR fungal isolates in lodgepole pine across 12 locations in British Columbia using 12 of 120 UBC primers (Sun *et al.* 1995).

Although the use of different primers in the two studies could potentially contribute to the contrasting pattern of RAPD variability, it is not supported by our results. First, the two sets of random primers, one from UBC and the other from Operon Technologies Inc. revealed a similar pattern of RAPD variability (Table 2-2). Second, we tested UBC Primer 482 which revealed polymorphisms for all individuals in all lodgepole pine populations studied by Sun *et al.* (1995), and all 30 of our isolates exhibited an identical RAPD pattern (results not shown). Third, divergence in results due to use of different primers is expected to decrease with increasing number of random primers and the two studies did screen a large number of random primers, 90 in this study and 120 in Sun *et al.* (1995). Thus, the variable results between the studies might reflect population differences.

Homogeneity of RAPDs in WGR fungal isolates sampled from lodgepole pine host might not be indicative of their genetic uniformity however. The small size of isolates sampled per population would bias downward the estimate of variability in this study. Two phenotypes, marker and null, are detectable at each RAPD. Since marker is dominant over the null, the population frequency of null is q^2 and marker is $p^2 + 2pq$, where $p + q = 1$. Thus, the probability for detecting segregating phenotypes in a population critically depends on q and the sample size. For example, with equal frequency of p and q, at 0.5, we would expect one null and three marker phenotypes in a sample of four isolates. When p is 0.9, we would expect one null and 99 marker phenotypes in a sample of 100 isolates. Since our sample sizes per population varied between 2 and 22, we would not expect to detect both null and marker phenotypes at a polymorphic RAPD unless the frequency was near 0.5. On the other hand, RAPD

fragments can be amplified either from an individual containing two copies of an allele or from an individual containing only one copy of the allele, which may also bias downward the estimate of the variability since the diploid tissues were used in this study.

The advantages of RAPDs are their rapidity, simplicity and the need for very small amounts of genomic DNA. To examine RAPD variability of the WGR fungus, we used the inner layer of binucleate aeciospores from a single gall to represent an isolate. In total we sampled 73 such WGR isolates from 13 locations, ranging from 2 to 22 isolates for each location. For some locations of jack pine origin, we detected RAPD variability within locations even with 5 isolates (Table 3-3). Yet, RAPD variability was not detected even with 22 isolates for one location in the southwest part of Alberta. Our results might reflect RAPD frequency variations among WGR populations. On the other hand, lack of variability among the WGR fungal isolates of lodgepole pine origin and among the isolates within some locations of jack pine origin might indicate the homogeneity among these isolates. This is consistent with remarkably homogeneous or identical isozyme structure of the WGR isolates accross wide areas of the United States observed by Vogler *et al.* (1991).

RAPD assays provided a fast way to survey differentiation among WGR fungal isolates at the DNA level. In this study, different types of isolates were identified by RAPDs, although homogeneity among isolates was observed in some locations. However, direct assessment of WGR fungal virulence might not be possible from the RAPD data. RAPD markers could be used to identify genetically divergent inoculum sources, which would maximize the effectiveness of host screening studies.

The focus of a RAPD assay is to obtain reproducible markers by optimizing the factors involved in template DNA amplifications. In this study, we screened two primer sets, one from Operon Technologies Inc. and the other from UBC. Repeated amplifications were conducted with some samples to optimize the amplification conditions and to select primers that gave sharp and reproducible RAPDs. Four primers from Operon Technologies Inc. and five primers from UBC were selected. They generated similar results with only minimal differences in revealing RAPD marker variation among the isolates of jack pine origin.

Random amplified polymorphic DNA (RAPD) enables a fast and inexpensive way to detect variations of nucleotide sequence for a number of organisms (Welsh and McClelland 1990; Williams *et al.* 1990). In RAPD analysis, co-migrating fragments are usually scored as the same markers. Co-migrating fragments have been shown to be homologous by segregation and hybridization studies of RAPD markers in many organisms (Williams *et al.* 1990; Hamelin *et al.* 1994).

In conclusion, RAPDs are useful DNA markers to discriminate among WGR fungal isolates and to study their geographic structure. However, the process that guides the coevolution of WGR fungi with their pine hosts is probably complex and poorly understood. There is still a need to discern the relative roles of historical factors, ecological factors and contemporary factors, such as gene flow, genetic drift, mating system, and selection in the present-day patterns of geographic diversity.

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Table 2-1. Collection of western gall rust isolates from jack pine and lodgepole pine hosts

Location Code	Isolate (n)	Host	Location	Longitude(^{0} N)	Latitude (^{0} W)
L1	3	Lodgepole pine	Tswassen, British Columbia	48.50	123.50
L2	3	Lodgepole pine	Christina Lake, British Columbia	49.00	118.50
L3	2	Lodgepole pine	Waterton, Alberta	49.05	113.55
L4	22	Lodgepole pine	St. Albert, Alberta	53.30	113.40
J1	2	Jack pine	North Star in Peace, Alberta	56.48	117.00
J2	3	Jack pine	Mariana Lake, Alberta	55.50	123.30
J3	5	Jack pine	Meadow Lake, Saskatchewan	54.25	109.00
J4	5	Jack pine	Dragline Lake, Manitoba	51.35	100.40
J5	8	Jack pine	Steeprock, Manitobia	51.25	98.30
J6	5	Jack pine	Belair, Manitobia	50.35	96.00
J7	5	Jack pine	Lonesand, Manitobia	49.15	96.25
J8	5	Jack pine	Sandiland, Manitobia	49.30	96.20
J9	5	Jack pine	Drgden List, Ontario	50.00	93.00

Table 2-2. Count of variable RAPDs detected in western gall rust isolates in jack pine and lodgepole pine hosts

Primers	Hosts		χ^{2a}
	Lodgepole pine (n=30)	Jack pine (n=43)	
UBC266-1230	0	43	72.98**
UBC283-1060	30	0	72.98**
UBC285-1900	0	43	72.98**
UBC285-520	30	0	72.98**
UBC289-1900	0	22	21.97**
UBC289-1550	0	3	2.17
UBC289-1150	30	3	61.75**
UBC290-850	0	43	72.98**
UBC290-520	30	0	72.98**
OPA02-950	0	35	46.89**
OPA02-510	0	21	20.57**
OPA07-2500	0	43	72.98**
OPA07-1070	0	40	61.75**
OPA07-970	0	2	1.43
OPA13-1580	30	0	72.98**
OPA13-1170	30	0	72.98**
OPA13-690	0	3	2.17
OPA14-960	0	43	72.98**

Note: n is number of isolates collected from lodgepole or jack pine hosts.
[a]Contingency chi-square test for homogeneity of RAPD count among hosts.
** Significant at $P \leq 0.01$.

Table 2-3. Count of variable RAPDs detected among geographic isolates of western gall rust fungus in jack pine host

RAPDs	Alberta		Saskatchewan			Manitoba			Ontario	
	J1 (n=2)	J2 (n=3)	J3 (n=5)	J4 (n=5)	J5 (n=8)	J6 (n=5)	J7 (n=5)	J8 (n=5)	J9 (n=5)	χ^{2a}
UBC289-1900	0	0	0	2	2	5	5	5	3	27.34**
UBC289-1550	0	0	0	1	2	0	0	0	0	7.53
UBC289-1150	0	0	0	1	2	0	0	0	0	7.53
OPA02-950	0	0	5	4	6	5	5	5	5	27.82**
OPA02-510	2	3	5	3	6	0	0	0	2	27.34**
OPA07-1070	2	3	5	4	6	5	5	5	5	7.53
OPA07-970	0	0	0	0	0	0	0	0	2	16.11*
OPA13-690	0	0	0	0	3	0	0	0	0	14.06

Note: n is number of isolates collected at different locations.

[a]Contingency chi-square test for homogeneity of RAPD count among locations.

** Significant at $P \leq 0.01$.

* Significant at $P \leq 0.05$.

Table 2-4. Analysis of RAPD variance among geographic isolates of western gall rust fungus in jack pine and lodgepole pine hosts

Source of variation	df	MSD[a]	Variance component	% total	*P*-value[b]
Between hosts	1	174.180	4.614	76.31	≤ 0.001
Among locations/host	11	4.588	0.871	14.40	≤ 0.001
Within locations	60	0.562	0.562	9.29	

[a] Mean squared deviations.
[b] Probability that the component estimate is different from zero by chance alone.

Table 2-5. Analysis of RAPD variance among geographic isolates of western gall rust fungus in jack pine host

Source of variation	df	MSD[a]	Variance component	% total	*P*-value[b]
Among locations	8	6.308	1.128	53.22	≤ 0.001
Within locations	34	0.991	0.991	46.78	

[a] Mean squared deviations.
[b] Probability that the component estimate is different from zero by chance alone.

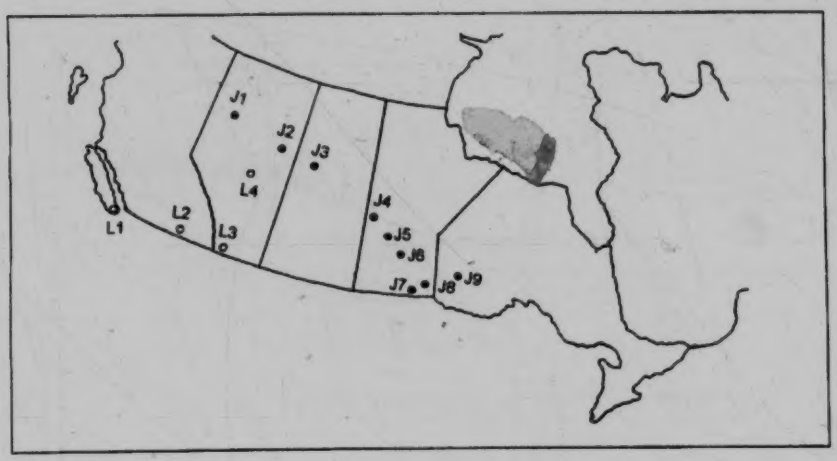

Figure 2-1. Geographic locations of western gall rust isolates collected from lodgepole pine (L1-L4) and jack pine (J1-J9) across Western and Central Canada.

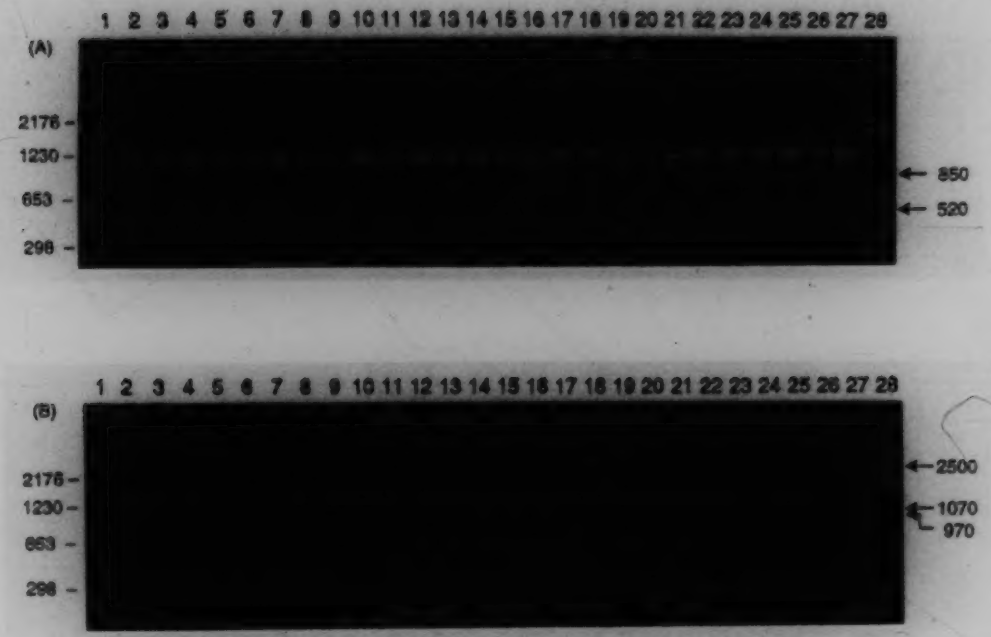

Figure 2-2. RAPD profiles of western gall rust isolates amplified with Operon primer OPA07 (A) and UBC primer UBC290 (B). Lane 1 and lane 28: molecular weight marker VI (Boehringer Mannheim). Lane 2-lane 9: RAPDs of isolates from lodgepole pine. Lane 10-lane 27: RAPDs of isolates from jack pine. Arrows indicate polymorphic RAPD fragments with their molecular sizes in base pairs on the right.

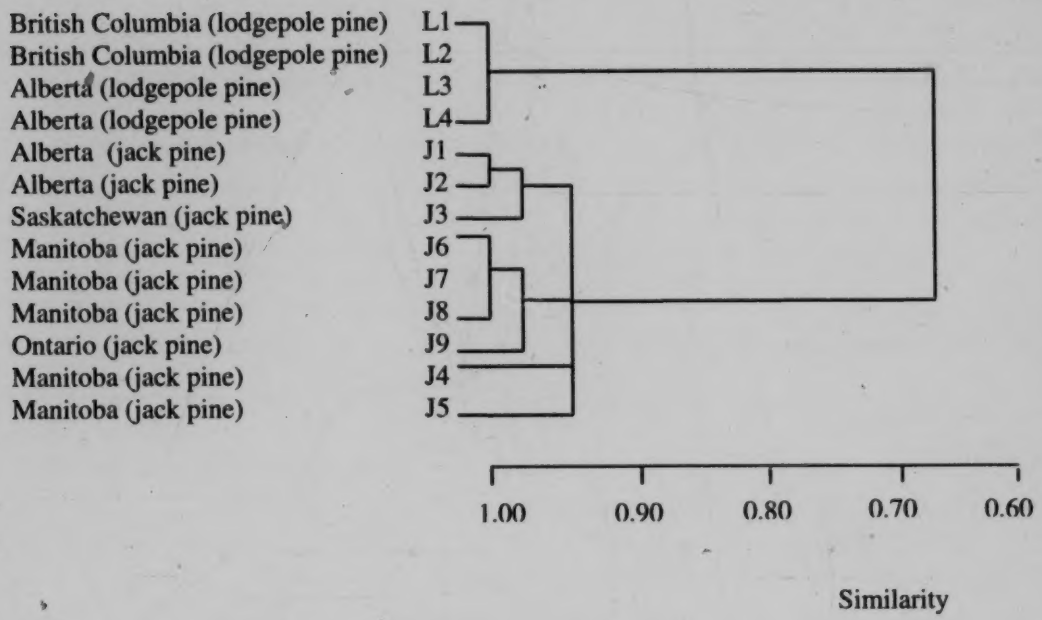

Figure 2-3. Dendrogram depicting RAPD similarity among geographic isolates of western gall rust fungus in jack pine and lodgepole pine hosts.

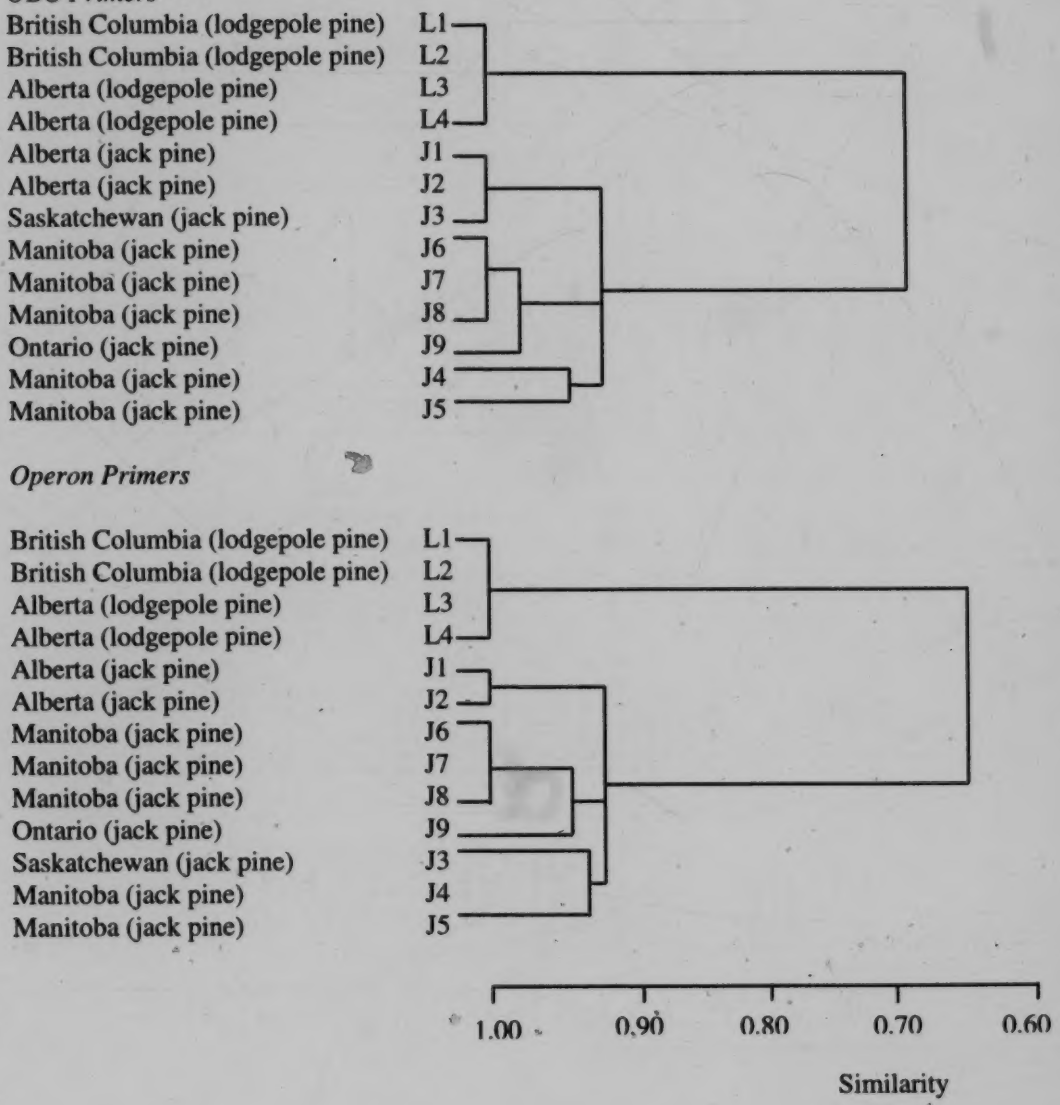

Figure 2-4. Dendrograms depicting similarity of western gall rust fungus isolates among locations based on cluster analysis of RAPDs amplified by primers from University of British Columbia Biotechnology Laboratory (UBC) and Operon Technologies Inc (Operon).

CHAPTER 3 EVALUATION OF RESISTANCE IN THREE ALBERTA LODGEPOLE PINE FAMILIES AGAINST TWO DIFFERENT WESTERN GALL RUST SPORE SOURCES IN GREENHOUSE

3.1 INTRODUCTION

Lodgepole pine is one of the major hosts of western gall rust (WGR) in North America. Damage caused by WGR has been reported frequently (Powell and Hiratsuka 1973; van der Kamp and Spence 1987; Bella and Navratil 1988; Yanchuk *et al.* 1988; Wu *et al.* 1996). Infections in the main stem of lodgepole pine by WGR can result in high mortality in young plantations or prevent trees from reaching merchantable size (Blenis *et al.* 1988). An abundance of galls on branches can also affect the growth of lodgepole pine significantly. Traditionally, efforts to control WGR fungal infection have focused on either containing the fungus (Tsuneda and Hiratsuka 1979; Blenis *et al.* 1988; 1996; Currie *et al.* 1995) or increasing genetic resistance in the host (Martinsson 1980; Yanchuk *et al.* 1988; Kojwang 1994). However, as the management of lodgepole pine plantations becomes intensive, the effective manipulation of both fungus and hosts must be considered as the prime objective.

An understanding of the range of variation in resistance in lodgepole pine and the interaction between WGR and lodgepole pine will provide fundamental information towards the effective manipulation of both the rust and the host. During the last two decades, the variation in resistance of lodgepole pine was investigated by both field observation and greenhouse study (Martinsson 1980; Yanchuk *et al.* 1988; van der Kamp 1088a;1988b; 1989; Blenis *et al.* 1993; Kojwang and van der Kamp 1991; Wu *et al.*

1996; Yang *et al.* 1997). All results indicated that lodgepole pine exhibited a remarkable variation in resistance to WGR.

The variability of virulence in the rust and the interaction between lodgepole pine and WGR were also examined by inoculating different provenances, families or clones of lodgepole pine with different WGR spore sources. Significant interactions between lodgepole pine and WGR spore sources were observed in some studies (van der Kamp 1988b; Kojwang and van der Kamp 1991) but not in others (van der Kamp 1989; Blenis *et al.* 1993). The non-significant interaction between lodgepole pine and WGR observed in some studies might be mainly due to the use of WGR spore sources with limited genetic differences or to lodgepole pine with limited resistance differences.

Selection of genetically divergent inoculum of WGR will maximize the effectiveness of host-pathogen interaction study. We have examined the geographic variability of WGR and identified two distinct types of WGR isolates based on the RAPD markers. The isolates from lodgepole pine hosts and the isolates of jack pine origin showed remarkably different RAPD patterns. In this study, three half-sib lodgepole pine families were inoculated with two WGR sources, one from Alberta collected from lodgepole pine, the other from Manitoba collected from jack pine. The objective was to evaluate the variation in resistance among the half-sib lodgepole pine families and examine the interaction between the families and genetically different WGR spore sources.

3.2 MATERIALS AND METHODS

3.2.1 Isolates collection

Seventy-three single galls were collected from lodgepole pine and jack pine hosts at 13 locations in May and June of 1994 as described in chapter 2. Galls were kept separate in separate paper bags and placed on an open bench, and were air dried before the extraction of spores. The spores from each gall were passed through a wire mesh screen (50-250 μm opening) to remove debris. Spores were then placed into glass vials containing silica gel, labeled and stored in a freezer (-15 ^{0}C) until use. The inner layer of spores for each gall were also collected and used to examine geographic variability using the RAPD technique. Those results were reported in Chapter 2.

Based on the results of our geographic variation study for WGR isolates, two single gall isolates that showed distinctive RAPD profiles were chosen for this study. One single-gall isolate was collected from Alberta, representing the spore source from lodgepole pine. The other single-gall isolate was collected from Manitoba, representing the spore source from jack pine.

3.2.2 Plant materials

Seeds from three open-pollinated families, A00588, A01013 and A01754, were used in this study. The three families were chosen from a long-term field and greenhouse screening experiment. The maternal trees of the three families were disease free in the natural stands and their half-sib families showed large phenotypic variance in resistance to WGR. The three lodgepole pine maternal trees originated from Alberta. Tree A00588 was 74 years of age at the time of seed collection in 1976 and near Wolfcreek, at 1036 m elevation, latitude 54^{0}36' N and longitude 119^{0}03' W. Tree A01013 was near Judy Creek, at 1070 m elevation, latitude 54^{0}26' N and longitude 115^0 34'W. It was 133 years of age at

the time of seed collection in 1977. Tree A01754 was 77 years of age at the time of seed collection in 1978 and near Windfall, at 1000 m elevation, latitude $54^0$01' N and longitude 116^0 34'W.

3.2.3 Experimental Design

Seeds of the three families were germinated in plastic trays (Ventblock ® 45, Beaver plastic Ltd.) in the greenhouse at the University of Alberta. Each tray contained 45 cavities (350 cm^2/cavity). Each cavity was filled with peat moss adjusted to pH 5-5.5 by the addition of dolomite lime. For a 113-L bale of peat moss, 358 g of lime was added. The medium was saturated with enough water to last until after sowing.

A two-way split plot design was employed in the study with two spore sources as whole plots and three families as subplots. The two spore sources were randomly assigned to the whole plots and the three families were randomly assigned to the subplots within each whole plot, with total replication of 90.

Seeds were directly sowed into the cavities on May 9, 1996 with 2 seeds in each cavity. For the first two weeks after sowing, deionized water was applied daily to maintain soil moisture. After two weeks, each cavity had 1-2 seedlings which were randomly thinned to one plant per cavity. The seedlings were grown under a natural photoperiod and controlled temperature of 20-25 ^{0}C. After thinning, watering was reduced to twice a week and a solution of complete fertilizer 20-20-20 (200 ppm) was supplied at two-week intervals in conjunction with seedling watering.

3.2.4 Inoculation procedures

Seedlings were inoculated with the two spore sources on July 15, 1996. The rust spore viability was tested on 0.2% agar on a microscope slide at 25^0C before inoculation. The tested germination rates for both spore sources were above 85%. Inoculation procedures followed the torn needle method developed by Myrholm and Hiratsuka (1993). Briefly, prior to the inoculation, the trays of seedlings were saturated with water and the seedlings were misted with distilled water. A single needle of a seedling was removed with a downward pull and dry spores were then applied directly on and around the small scar left by the needle using a small paintbrush. After all seedlings were inoculated, they were lightly misted with atomized distilled water. Inoculated seedlings were covered with a plastic sheet to maintain high humidity for spore germination and infection, and kept at 15^0C in a dark growth room. The seedlings were uncovered after 48-h and returned to normal growing conditions.

3.2.5 Evaluation of disease infections

Disease infections were evaluated six months after inoculation. The infection level was rated based on evaluation criteria developed at the Northern Forestry Centre, Canada (Klein *et al.*1991). A rating of 0 represents a lack of symptoms, whereas a rating of 5 represents a globose gall. Ratings of 1, 2, 3 or 4 were given to the seedlings according to their gall appearance, from slight sign of infection to partial gall.

3.2.6 Statistical analysis

Analyses of variance (ANOVA) were used to examine the family effect, spore source effect and their interaction. The disease rating data were transformed by $\log_{10}$(1+disease

rating) to approach a normal distribution. GLM procedure (SAS Institute Inc.) was performed with a mixed model for the disease infection trait. The mixed linear model was:

$$Y_{ijh} = \mu+S_i+F_j+SF_{ij}+R_h+SR_{ih}+E_{ijh} \quad [1]$$

Where Y_{ijh} = the observation of the jth family under the treatment of ith spore sporec in the hth replication,

μ = overall experimental mean,

S_i = spore source effect (fixed),

F_j = family effect (fixed),

SF_{ij}=effect of family by spore source interaction (fixed),

R_h = replication effect (random),

SR_{ih}=effect of spore source by replication interaction (random),

E_{ijh}=residual error.

Type III mean squares were used in all-F tests (Table 3-1). When appropriate error terms were not directly available, Satterthwaite (1946) approximation method was used and the degrees of freedom was derived as shown in Milliken and Johnson (1984). Differences among means of treatment levels were tested with Duncan's multiple mean comparison method.

3.3 RESULTS

3.3.1 Family effect

Variance analysis results indicated that the family effect was significant at 0.001 level (Table 3-2). Overall, the average infection rating for the three families was 2.308 (Table 3-3). However, the three families showed significant variability in resistance to the WGR isolates. Family A01013 was less infected by the WGR, with the average infection rating of 1.825, while family A00588 was the most susceptible to the disease, with the average infection rating of 2.782. Family A01754 was ranked between them, with the average infection rating of 2.326. The average infection ratings were significantly different from each other at 0.05 level (Table 3-3).

3.3.2 Spore source effect

Spore source effect was also significant at 0.001 level (Table 3-3). The local spore source, representing the spore source from lodgepole pine, was less virulent to the three families, with the average infection rating of 2.056. The exotic spore source, which represents the isolate from jack pine, however was more virulent to the three families with the average infection rating of 2.559 (Table 3-3).

3.3.3 Family by spore source interaction

Significant family by spore source interaction was detected at 0.0019 level (Table 3-2). By examining the resistance performances of the three families across the two WGR spore sources, it was found that the three families demonstrated different responses to the two spore sources. The three families infected by the exotic spore source showed significant variation in resistance to the isolate, while no significant difference in resistance was detected among the families infected by the local spore source (Table 3-3).

The three lodgepole pine families showed responses of infection to the two spore sources. Family A00588 and Family A01754 tended to be heavily infected by the rust of jack pine origin while Family A01013 showed the most resistance. Family A00588 was the most susceptible to the rust of jack pine origin, with an average infection rating score of 3.517. Family A01013 was the most resistant to the rust source of jack pine origin, with an average infection rating score of 1.618. Family A01754 was intermediate, with an average infection rating score of 2.541.

3.4 DISCUSSION

Infection responses of lodgepole pine families inoculated with different WGR isolates could provide insight into the host-pathogen system. In this study, we inoculated three lodgepole pine families with two genetically divergent WGR spore sources and found significant family, spore source and family by spore source interaction effects. Family A00588 and A01754 were significantly more resistant to the lodgepole pine spore source than to the jack pine spore source, while family A01013 was more susceptible to lodgepole pine spore source than to jack pine spore source. Different responses in resistance of lodgepole pine families to different WGR isolates were also detected in other studies. van der Kamp (1988b) found two coastal provenances of lodgepole pine were significantly more resistant to the interior spore sources than to the coastal spore source. The differentiation in resistance to different WGR isolates in lodgepole pine might reflect a strong co-evolutionary history between the host and the pathogen.

The jack pine spore source appeared to be more pathogenic to the three lodgepole pine families, and the three lodgepole pine families demonstrated great differentiation in

resistance to jack pine spore source. However, the three lodgepole pine families showed almost the same level of resistance to the lodgepole pine spore source. These results may suggest that jack pine spore sources have an increased virulence to lodgepole pine families, while the co-evolution between the lodgepole pine families and the lodgepole pine spore source has reached a certain stage of equilibrium. The introduction of exotic inoculum or of other hosts seems to disturb the existing equilibrium between the host and the pathogen.

However, rust sources from different hosts may not necessarily show pathogenicity variation if they have similar origins or the host preference is not well defined. van der Kamp (1989) tested the resistance of lodgepole pine against two rust spores collected from Scots pine and lodgepole pine in Vancouver. However, the two rust sources exhibited no significant difference in their infections to lodgepole pine, indicating that WGR races specific to Scots pine may not be developed (van der Kamp 1989).

A mixed system, in which both horizontal and vertical effects occur, was proposed for the lodgepole pine-WGR pathosystem by Kojwang and van der Kamp (1991). In our study, partial galls were observed throughout the experiment, which might indicate the quantitative resistance of lodgepole pine to WGR. The distribution of resistance performances among half-sib families demonstrated remarkably different patterns among the three lodgepole pine families (Figure 3-1). Resistant families tended to have more non-infections while susceptible families contained more infections of complete galls and partial galls. This result might suggest that the resistance of lodgepole pine to WGR be conditioned by several major genes, complemented by some minor genes. The more major resistance genes present, the more non-infections will be expected, as in family

A01013 against WGR from Alberta and Manitoba sources. Consequently, the distribution pattern in family A01754 against the rust from Manitoba might suggest the presence of less major resistance genes in the family. In contrast with family A01013, the distribution pattern of infection in Family A00588 against rust of Manitoba source strongly suggests resistance genes with only a minor effect. However, a further genetic study is needed to draw final conclusions.

Great variations in resistance within each of the three half-sib families were observed, not only in resistance to lodgepole pine spore source but also to jack pine spore source. This result suggests that a great potential for selection of resistance in lodgepole pine to WGR also exist at the individual level.

Artificial inoculation of lodgepole pine with genetically different WGR isolates proved an effective way to assess the pathogen-host relationship. Molecular markers such as RAPDs can be used to characterize the isolates and provide information for proper selection of isolates in host-pathogen studies. Further study using more lodgepole pine families and more WGR isolates with genetic differences will provide a better understanding of the pathogen-host system, increasing the effectiveness of manipulation of both the host and the pathogen.

The great variations in resistance among individuals within each of the three lodgepole pine families confirmed previous results that resistance genes in maternal trees are heterozygous and therefore segregated among their offspring. This allows the detection of resistance genes in the maternal trees by RAPD markers using their half-sib families.

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Table 3-1. Degrees of freedom and expected mean squares in analysis of variance to determine the spore source and family effects (mixed model)

Source	df	Type III expected mean squares
R	89	Var(error b) + 2.818 Var(error a) + 5.6359 Var (R)
S	1	Var(error b) + 2.7821Var(error a) + Q(S)
Error a	89	Var(error b) + 2.818 Var(error a)
F	2	Var(error b) + Q(F)
S x F	2	Var(error b) + Q(S x F)
Error b	328	Var(error b)

Note: R, replication; S, spore sources; F, family; Sx F, spore source by family interaction.

Table 3-2. Analysis of variance of spore source and family effects based on the $\log_{10}$ (1+disease rating) values

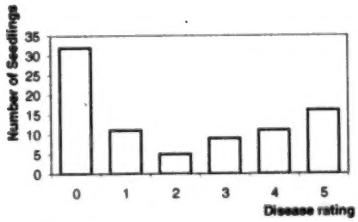
Source[a]	df	Type III Sum of Squares	Mean Square	F value	Pr > F
R	89	47.429	0.533	1.081	0.3039
S	1	6.234	6.234	10.219[b]	0.0019
Error a	89	54.423	0.612		
F	2	14.875	7.438	15.087	0.0001
S x F	2	11.563	5.782	11.728	0.0001
Error b	328	161.693	0.493		

Note: [a] R, replication; S, spore source; F, family; Sx F, spore source by family interaction.
[b] the error term synthesized by Satterthwaite's approximation was 0.610 with df of 90.86.

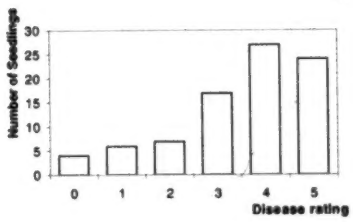
Table 3-3. The means of disease rating values of three lodgepole pine families across two spore sources

Family	Spore Source Local (Alberta)	Spore Source Exotic (Manitoba)	Mean
A00588	2.047a	3.517b	2.782e
A01013	2.011a	1.618c	1.825f
A01754	2.110a	2.541d	2.326g
Mean	2.056	2.559	2.308

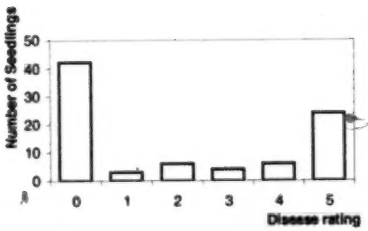
Note: same letters indicate no difference at 0.05 level. Different letters indicate significant difference at 0.05 level.



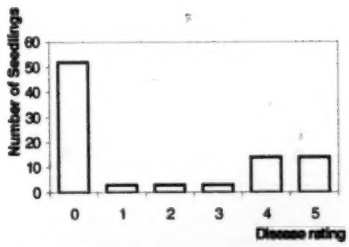
Family A00588 vs. Lodgepole pine Pine Spore Source



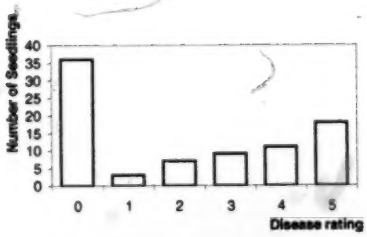
Family A00588 vs. Jack Pine Spore Source



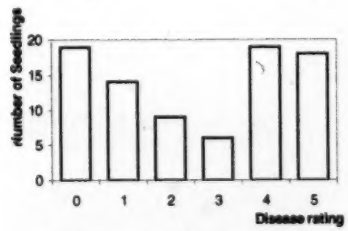
Family A01013 vs. Lodgepole Pine Spore Source



Family A01013 vs. Jack Pine Spore Source



Family A01754 vs. Lodgepole Pine Spore Source



Family A01754 vs. Jack Pine Spore Source

Figure 3-1 Distribution of disease scores in lodgepole pine families A00588, A01013 and A01754 against western gall rust from Alberta and Manitoba sources

CHAPTER 4 CONSTRUCTION OF HIGH DENSITY GENOMIC MAPS IN THREE LODGEPOLE PINE FAMILIES USING RANDOM AMPLIFIED PLOYMORPHIC DNA (RAPD) MARKERS

4.1 INTRODUCTION

Genetic linkage maps have many potential applications in forest genetics and tree improvement research (Neale and Williams 1991; Nelson et al. 1993; Staub *et al.* 1996). The genome organization of forest trees could be better understood with the availability of genetic linkage maps. In population and evolutionary studies, unbiased estimates of certain parameters could be accomplished by choosing appropriate markers from different linkage groups. Comparison of linkage maps among species may provide some information regarding their phylogenetic relationships. Marker-assisted selection (MAS) in tree improvement would be more effective with knowledge of the linkage relationships between genetic markers and traits of interest.

The most important application of genetic linkage maps in forest genetics and tree improvement research is the identification and localization of genes controlling quantitative traits of interest. A complete genomic map with densely spaced genetic markers is essential for systematic and accurate mapping of quantitative trait loci (Lander and Botstein 1989). In the last few years, detection and mapping of quantitative trait loci for commercially important traits based on genetic linkage maps has been successful in several forest species (Groover *et al.* 1994; Bradshaw and Stettler 1995; Grattapaglia *et al.* 1995).

Most of the earlier studies in genetic linkage analysis and genetic map construction in forest trees were performed using isozyme markers (Guries *et al.* 1978; Rudin and Ekberg 1978; Adams and Joly 1980; Conkle 1981; Eckert *et al.* 1981; Neale and Adams 1981; El-Kassaby *et al.* 1982; King and Dancik 1983; Cheliak and Pitel 1985; Harry 1986; Muona *et al.* 1987; Na'iem *et al.* 1993) and later using restriction fragment length polymorphisms (Devey *et al.* 1994; Bradshaw *et al.* 1994). However, the limited number of loci in isozyme markers and the time-consuming RFLP technology impeded the construction of linkage maps with reasonable coverage of the genome for many species.

The recently developed random amplified polymorphic DNA (RAPD) markers (Welsh and McClelland 1990; William *et al.* 1990) accelerated the construction of genetic linkage maps in forest trees. The potential abundance of RAPD markers and the rapidity and simplicity of the RAPD technique compared to RFLP analysis make it desirable to use in construction of high density genetic linkage maps in forest trees. Since RAPD analysis only needs a minimal amount of DNA for each reaction, the technique is very attractive for construction of genetic maps in conifers when the haploid megagametophytes are used to generate the genetic markers. Tulsieram *et al.* (1992) mapped 47 RAPD markers into 12 linkage groups covering 873.8 cM in *Picea glauca*. Nelson *et al.* (1993) placed 73 RAPD markers onto 22 linkage groups and pairs in *Pinus elliottii*, covering a distance of approximately 782 cM. In *Picea abies*, 185 RAPD markers were mapped into 17 major linkage groups with a total distance of 3584 cM (Binelli and Bucci 1994). Grattapaglia and Sederoff (1994) constructed two genetic linkage maps for *Eucalyptus grandis* and *Eucalyptus urophylla* using RAPD markers. The genetic linkage map for maternal *Eucalyptus grandis* contains 240 RAPD markers in

14 linkage groups with a distance of 1552 cM, while linkage map for paternal *Eucalyptus urophylla* contains 251 markers in 11 linkage groups covering a distance of 1101 cM. In *Pinus taeda*, 189 RAPDs were mapped to 16 linkage groups with a total distance of 1726.7 cM (Wilcox 1995). More recently, Yazdani *et al.* (1995) reported a genetic linkage map for *Pinus sylvestris* with 261 RAPD markers in 14 linkage groups covering a distance of 2638.6 cM.

Lodgepole pine (*Pinus contorta* Dougl. var. *Latifolia* Engelm.) has achieved significant stature as a commercial forest species, not only in North America but also throughout much of Northern Europe (Wheeler and Critchfield 1985). During the last several decades, genetic studies and tree improvement programs for the species have mainly focused on population structure (Critchfield 1957; Yeh and Layton, 1979; Wheeler and Guries 1982; Yeh *et al.* 1985; Yang and Yeh 1993; Yang *et al.* 1996), and progeny test and selection (Lines 1976; Rehfeldt 1985; Ying *et al.* 1985; Dhir and Barnhardt 1993). The genetic linkage map data for the species however are limited. Conkle (1981) placed 20 isozyme markers into 4 linkage groups in lodgepole pine sampled from Sierra Nevada. The 4 linkage groups covered a distance of approximately 170 cM.

The objective of this study was to construct high density genomic maps in three half-sib lodgepole pine families using RAPD markers. The primary goal was to generate three maps containing a reasonable number of markers that will facilitate the identification of quantitative trait loci for resistance to western gall rust (WGR) and other commercially important traits in lodgepole pine, and provide genetic markers for choice in population and evolutionary studies of the species.

4.2 MATERIALS AND METHODS

4.2.1 Plant materials

Open-pollinated seeds were collected from three plus trees A00588, A01013 and A01754. Those trees were resistant to WGR in natural stands and their half-sib families showed large phenotypic variances in resistance to WGR in a long-term field and greenhouse screening. The three lodgepole pine maternal trees originated from Alberta. Tree A00588 was 74 years of age at the time of seed collection in 1976 and near Wolfcreek, at 1036 m elevation, latitude $54^0$36' N and longitude $119^0$03' W. Tree A01013 was near Judy Creek, at 1070 m elevation, latitude $54^0$26' N and longitude 115^0 34'W. It was 133 years of age at the time of seed collection in 1977. Tree A01754 was 77 years of age at the time of seed collection in 1978 and near Windfall, at 1000 m elevation, latitude $54^0$01' N and longitude 116^0 34'W. The seeds were germinated in the greenhouse and the megagametophytes were obtained by removing them from the extending cotyledonary needles of the germinating seedlings prior to natural abscission. The seedlings were grown in the greenhouse for future QTL analysis. The megagametophytes were stored at -20 ^{0}C until required for DNA extraction.

4.2.2 DNA extraction

Total DNA was extracted for each megagametophyte using a method modified from that described by Lee and Taylor (1990). Briefly, one megagametophyte and 700 µl extraction buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 3% SDS, 1% 2-Mercaptoethanol) were homogenized with a pellet pestle by hand in a 1.5 ml

microcentrifuge tube and then the mixture was incubated for 1 hour at 65 ^{0}C. After incubation, 700 µl phenol:chloroform (Applied Biosystems Inc.) solution was added. The tube was vortexed briefly, then centrifuged for 10 min at 13,000 rpm. The upper phase was purified again with 700 µl chloroform:octanol (24:1, v:v). The supernatant was precipitated with a 10% final volume of 3.0 M sodium acetate (pH 5.6) and 2-2.5 volume of 90% cold ethanol. The pellet was rinsed with 70% ethanol, air dried and resuspended in 45 µl TE buffer (10mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Finally, 5 µl of DNase-free RNase A (100 µg/ml, Boehringer Mannheim) was added and the solution was incubated for 1 hour at 37 ^{0}C. This method usually yielded 1-2 µg of DNA per megagametophyte.

4.2.3 DNA amplification

Each DNA amplification was carried out in a volume of 20 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl. 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP, 0.20 µM random primer and 2 ng of DNA sample together with 0.70 unit of AmpliTaq DNA polymerase (Perkin Elmer). Amplifications were performed with MicroAmp reaction tubes in GeneAmp PCR system 9600 (Perkin Elmer), programmed for 40 cycles of 30 seconds of denaturing at 92 ^{0}C, 30 seconds of annealing at 36 ^{0}C, and 1 min of extension at 72 ^{0}C after an initial denaturation of 2 min at 92 ^{0}C. The program was followed by an extension phase at 72 ^{0}C for 8 min. The RAPD products were separated by electrophoresis on 1.5% agarose gels in TBE buffer at 75 v for 3.5 hours. Gels were photographed under illumination with UV light after ethidium bromide staining.

The random primers used in this study were 10-base, oligonucleotide primers obtained from University of British Columbia (numbers 1-800) and from Operon Technologies. After initial screening of 840 random primers against eight megagametophyte DNA samples for each family, 110 primers were selected based on their performance in generating segregating, sharp and consistent markers for family A00588. Ninety-six primers were selected for family A01013 and 104 primers were chosen for family A01754 based on the same criteria. Among the primers selected for the three families, 91 of them were common primers for the all three families. A set of 90 megagametophyte DNAs from family A00588 were then amplified with each of the 110 selected primers. DNAs of 60 megagametophytes from family A01013 were amplified with the 96 selected primers. Another set of 60 megagametophytes DNAs from family A0174 were also amplified with the 104 primers for that family.

4.2.4 RAPD marker analysis

Segregating RAPD fragments were scored either as "1" (presence) or as "0" (absence) for each megagametophyte. The molecular sizes of RAPD fragments were estimated by comparison with DNA molecular weight marker VI (Boehringer Mannheim) using DNA ProScan program (DNA ProScan, Inc.) The RAPD fragments were assigned names based on their primer identities and molecular sizes. The RAPD markers were then tested for goodness of fit to a 1:1 Mendelian ratio using chi-square statistic at 5% significance level. The RAPD markers that were significantly distorted from the 1:1 ratio were excluded prior to the linkage analysis.

4.2.5 Linkage analysis and map construction

Linkage analysis and map construction was performed with Mapmaker for Macintosh V2.0 (Lander *et al.* 1987). Raw data were prepared as an F2 backcross data file with all RAPD markers duplicated and recoded inversely to allow the Mapmaker program to identify linkages between markers in both coupling and repulsion phases. We first used two-point analysis to assign the entire set of markers into possible linkage groups with a LOD score of at least 3.0 and a maximum recombination fraction of 0.40. Within each of the possible linkage groups, we then performed three-point and multi-point analyses to determine the most likely orders. Finally, matrix correlation analysis was used to confirm the map orders. The genome size G was estimated in cM by G=N(N-1)X/K] (Hulbert *et al.* 1988). N is the number of markers used in the linkage analysis. X is the maximum distance between two markers for which the expected value of the LOD score is 3.0. K is the observed number of the linkage pairs for which the LOD scores exceed a value of 3.0.

4.3 RESULTS

4.3.1 Genomic mapping in family A00588

The 110 primers generated 328 segregating fragments (an average of 2.98 fragments per primer). The sizes of fragments ranged from 260 to 3080 base pairs. Two hundred and ninety one of these fragments were found to be consistent across different amplifications and were scored as RAPD markers for further analysis. Figure 4-1 represents one of the RAPD profiles.

Chi-square analysis indicated that 18 of the 291 RAPD markers were significantly distorted from a 1:1 ratio and consequently were excluded from mapping. Two hundred

twenty-five of the remaining 273 RAPD markers were then mapped to 16 main linkage groups (Figure 4-2, Figure 4-3). Other markers were found to be either unlinked or linked in pairs and triplets. By relaxing the LOD score and increasing the recombination fraction, some of those markers could be mapped onto main linkage groups. However, their mapping positions were not accepted since they were only loosely linked onto the main linkage groups at recombination fractions near 0.50.

The 16 main linkage groups cover a distance of 3517.5 cM. The length of each linkage group ranged from 92.2 cM to 468.9 cM with an average distance of 15.63 cM between two adjacent markers. The genome size was estimated to be 3680.5 cM for an LOD score of 3.0. The 16 linkage groups of our genetic map with 225 RAPD markers cover 95.6% of the genome.

4.3.2 Genomic mapping in family A01013

In family A01013, the 96 primers generated 268 segregating fragments (an average of 2.79 fragments per primer) with sizes ranging from 305 to 2680 base pairs. Two hundred and forty one of these fragments were consistent across different amplifications and were scored as RAPD markers for further analysis. Figure 4-1 represents one of the RAPD profiles.

Twenty-one of the 241 RAPD markers were found to be significantly distorted from a 1:1 ratio by chi-square analysis and consequently were excluded from mapping. One hundred and seventy two of the remaining 220 RAPD markers were mapped onto 16 linkage groups (Figure 4-4, Figure 4-5). Other markers were found to be either unlinked or linked in pairs or in triplets. By relaxing the LOD score and increasing the

recombination fraction, some of those markers could be eventually mapped onto main linkage groups. However their mapping positions were not accepted since they were only loosely linked onto the main linkage maps at recombination fractions near 0.50.

The 16 main linkage groups cover a distance of 3496.0 cM. The length of each linkage group ranged from 63.9 cM to 465.0 cM with average distance of 20.33 between two adjacent markers. The genome size was estimated to be 3620.6 cM for an LOD score of 3.0. The 16 linkage groups of this genetic map with 172 RAPD markers cover about 96.6% of the genome.

4.3.3 Genomic mapping in family A01754

In family A01754, 104 primers generated 288 segregating fragments ranging from 310 to 2750 base pairs. On average each primer generated 2.77 segregating fragments. Two hundred and seventy one fragments showed a consistent pattern across different amplifications and were scored as markers for further analysis.

Chi-square analysis detected that 14 of the 271 RAPD markers were distorted significantly from a 1:1 ratio and thus were excluded from mapping. Of the remaining 257 RAPD markers, 234 markers were mapped onto 17 linkage groups (Figure 4-6, Figure4-7). Other markers remained unlinked or linked in pairs and triplets. Those markers could be mapped onto the main linkage maps by relaxing the LOD score and increasing the recombination fraction, however they were then only loosely linked onto the main linkage groups at recombination fraction near 0.50, and consequently were not placed on the main linkage groups.

The 17 main linkage groups cover a distance of 3398.2 cM. The length of each linkage group ranged from 46.5 cM to 527.6 cM. The average distance between two adjacent markers was 13.98 cM. The genome size was estimated to be 3580.4 cM for a LOD score of 3.0. The 17 main linkage groups covers about 94.9% of the genome.

4.3.4 Comparison of genomic maps across the three families

Table 4-1 summarizes the putative common RAPD markers identified across the three lodgepole families prior to the map construction. Forty putative common RAPD markers were found between family A00588 and family A01013. Forty six putative common RAPD were detected between family A00588 and family A01013 while thirty four putative common markers were identified between family A01013 and family A01754. Among them, ten RAPD markers were found common across all three families. However some of these putative markers were not mapped onto the main linkage groups. The putative RAPD markers which were mapped onto the linkage maps were indicated by either a "*" or "**". A" **" indicates the RAPD markers which were common across the three families. A "*" indicates the RAPD markers which were common between two families. It was found that thirty-two of the forty common marker pairs between family A00588 and family A01013 were mapped onto the main linkage groups. Forty-three of forty-six common marker pairs between family A00588 and family A01013 were mapped onto the main linkage groups while twenty-four out of thirty-four common marker pairs between family A01013 and family A01754 were placed onto their main linkage groups. Eight common markers of all three families were mapped onto the main linkage groups.

By examining the positions of these putative common RAPD markers in the linkage maps of the three lodgepole pine families, we have identified twelve parallel linkage groups, each containing several common RAPD marker pairs between families. The twelve putative parallel linkage groups are presented in twelve figures from Figures 4-8 to 4.19.

It was found that seventy-two of the seventy-nine linked common markers between families or linked common markers across the three families were still linked in their corresponding parallel linkage groups. However, seven pairs of common markers were not placed in their corresponding parallel linkage groups. Such markers were identified and indicated in Table 4-1 by "m".

Marker Utts-1305 and Uwrs-1415 were two of the twenty-three common markers mapped between family A00588 and family A01013. However, the two markers were not mapped onto the parallel linkage groups between the two families. Marker Utts-1305 was placed onto parallel linkage group 8 of family A00588, but in family A01013, the marker was placed in parallel linkage group 4. Marker Uwrs-1415 was located in parallel linkage group 3 of family A00588. However, the markers were mapped in parallel linkage group 12 in family A01013. Three of thirty-three common linked markers between family A00588 and family A01754, Uelr-530, Ufsj-2100 and Utjb-520 were also not mapped onto their corresponding parallel linkage groups. Marker Uelr-530 was placed in the parallel linkage group 1 of family A00588 and parallel linkage group 9 of family A01754. In family A00588, marker Ufsj-2100 was mapped in parallel linkage group 8 while in family A01754 it was located in parallel linkage group 1. The third common marker, Utjb-520, was mapped both in parallel linkage group 6 of family A00588 and in parallel

linkage group 9 of family A01754. Marker Uwzf-800 was one of the sixteen common linked markers between family A01013 and family A01754. However the marker was mapped onto different parallel linkage groups of the two families, located in parallel linkage group 10 of family A01013 and parallel linkage group 5 of family A01754.

In total, seven of eight common linked markers across all the three families were mapped onto the same parallel linkage groups of all three families. One common marker was placed in each of parallel linkage groups 1, 4, 9, 10, 12 and two markers in parallel linkage group 7. One common linked marker, Uttl-1130, was not mapped onto the same parallel linkage groups of the three families. It was located in group 2 of both family A00588 and family A01013, but in group 10 of family A01754.

4.4 DISCUSSION

In conifers, heterozygous loci in a maternal tree will segregate in a 1:1 ratio in the haploid megagametophytes. The RAPD technique can detect the segregating loci among the haploid megagametophytes of a sib family unambiguously, which makes the technique advantageous to generate genetic markers from megagametophyte DNAs. The analysis of cosegregating RAPD markers among the megagametophytes facilitates linkage map construction. The second advantage of using RAPD markers is that the RAPD procedure requires only a small amount of DNA template for each reaction, which enable a sufficient number of markers from the megagametophytes to be obtained for mapping. In lodgepole pine a single megagametophyte harvested from a germinating seedling usually weighs 2.0 mg and yields about 1-2 µg of template DNA. By the RAPD procedure, approximately 100 reactions are expected for each single megagametophyte,

which will produce about 300 segregating markers. We have constructed genetic linkage maps using RAPD analysis on megagametophytes harvested from germinating seedlings for three lodgepole pine half-sib families. The genomic maps of the three lodgepole pine families contain an average of 210 RAPD markers with an average distance of approximate 15 cM between two adjacent markers and cover up to 96.6% of their genomes.

The size of the conifer genome has been estimated for some conifer species based on genetic maps. Neale and Williams (1991) estimated that the genome of pine species was approximately 2500 cM each. Nelson *et al.* (1993) reported that the genome size for *Pinus elliottii* was between 2880 to 3360 cM. Gerber and Rodolphe (1994) provided several estimates of genome size for *Pinus pinaster*, ranging from 1085 to 3128, using different approaches. The genome size of loblolly pine was estimated to be 2150 cM (Wilcox 1995). In a genetic map of *Picea abies*, the total map length was 3584 cM (Binelli and Bucci 1994). More recently, Remington *et al.* (1998) estimated that the genome length of loblolly pine was approximately 1700 cM based on the linkage map containing 508 AFLP markers. In this study, the genome sizes of the three lodgepole pine families ranged from 3580.4 to 3680.5 cM with an average of 3627.2 cM. Apparently, our estimates of genome length were greater than that of other pine species. The variation in estimates of genome size in those conifer species may simply reflect the biological phenomena of heterogeneity of recombination rates among cytodemic species, and/or differences in analytical approaches used to estimate the map length (Wilcox 1995). Simulation studies show that the Hulbert estimator that I used tends to overestimate

genome length (Chakravarti *et al.* 1991). The upward bias may be due in part to ignoring the effect of chromosome ends (Remington *et al.* 1998).

However, spurious linkages on the genetic linkage maps might also inflate the estimates of map length. The spurious linkages could be due to genotyping errors and false linkages of genetic markers by chance. Although generating consistent segregating markers has been the priority in this study, there were some faint bands (about 10%) that were ambiguous for some individuals. This could be one source of genotyping errors. We used a LOD score of 3.0 to group the markers. This implies a 1,000 times likelihood of detecting linkage between two markers over the null hypothesis that the two markers are unlinked. Thus, the chance of including a spurious linkage in the genetic map is 1×10^{-3}. Using more stringent LOD scores greater than 3.0 in the initial grouping of markers will definitely reduce the likelihood of detecting false linkages (David O'Malley, personal communication).

The assignment of genetic markers to linkage groups depends on the recombination fractions among those markers. In conifers, heterozygous loci are subject to 1:1 segregation among the haploid megagametophytes of a maternal parent tree, and consequently the RAPD fragments are expected to segregate in the same ratio. The recombination fractions were then estimated based on the analysis of cosegregating RAPD markers. The sample size of haploid megagametophytes is related to the accuracy of recombination fraction estimation and thus affected the placement of RAPD markers onto linkage groups. Allard (1956) proposed that for a given sample size of n, the standard error for a recombination fraction p can be expressed as $(p(1-p)/n)^{1/2}$ for backcross type data. Our sample sizes of 90 and 60 gave the estimate of a standard error

of 0.053 and 0.064 respectively for a maximum recombination fraction of 0.50, which is acceptable for a primary genome map. To achieve a much lower standard error of recombination fraction, the sample size must be increased greatly. For example a sample size of 2500 is required to ensure a maximum standard error of 0.010.

The number of RAPD markers scored also plays an important role in saturating the genetic map. The minimum number of randomly distributed markers required to cover a proportion p of a genome size of k at a maximum distance 2×c between two adjacent markers is given by n=[log(1-p)/log(1-2c/k)] (Lange and Boehnke 1982). Obviously, our genetic maps for the three families were not well saturated with an average of 210 RAPD markers. In family A00588, there is a total of 17 gaps that exceed 30 cM distance or 27% recombination frequency between two adjacent markers. The largest gap was observed in group 8 that has a distance of 37.9 cM or 32% recombination frequency. In family A01013, 19 gaps between two adjacent markers exceed 30 cM of map distance. The largest gap was found in linkage group 1 at 44.8 cM or 35.7% recombination frequency. Fourteen gaps between two adjacent markers in family A01754 exceed 30 cM of map distance. The largest one was in linkage group 12 with distance of 57.5 cM or 40.9% recombination frequency. It is conceivable that mapping additional RAPD markers will eventually fill the larger gaps in the maps and join smaller linkage groups into larger ones. We estimated that to cover 95% of a genome with a genome size of 3700 cM at a maximum distance of 30 cM between any two adjacent markers (c=15) in lodgepole pine, about 368 markers are required. However serious regional suppression of meiotic recombination due to inversions or other chromosomal variations could increase upward the number of markers required to saturate a genetic map.

The three genomic maps were remarkably conservative in terms of their common markers. Most of the linked common marker pairs (91.1%) remained linked in parallel linkage groups. Only seven common marker pairs (8.9%) among families were mapped onto different parallel linkage groups. The "mismapped" common marker pairs might be due to the sampling error or false linkages on the linkage maps.

Twelve parallel linkage groups identified in this study correspond to the number of haploid chromosomes in lodgepole pine (2N=24). It is observed that the lengths of linkage maps differed greatly among the three lodgepole pine families in some parallel linkage groups, which might suggest the absence of some linkage sections due to the limited number of RAPD markers used, or might reflect difference of chromosome size among families.

Although the putative RAPD common markers among the three families were conservative in their linkage relationships, the order or distance between the linked putative common marker pairs varied as shown in Figure 4-8 to Figure 4-19. In genomic map construction, assigning the order of markers to the linkage group has been one of the persistent problems, especially when there are a large number of markers. With MAPMAKER, the procedure to generate a marker order is to compare the likelihood odds with an alternative order, and chose the one with the highest likelihood. The conventional approaches to order a large set of markers begin with the selection of a subset of markers and place the other markers by multipoint analysis. The limitation of such ordering process lies the inherent possibility of generating some false linkage orders, especially when large numbers of markers are being ordered, and is the function of the LOD score used in grouping and ordering of markers.

The potential applications of genomic maps in forest genetics and tree improvement have been recognized (Neale and Williams 1991; Nelson *et al.* 1993). The most exciting utility of genomic maps is the identification and mapping of quantitative trait loci for commercially important traits, as demonstrated in many forest species (Groover *et al.* 1994; Bradshaw and Stettler 1995; Grattapaglia *et al.* 1995). One of our intentions in constructing genetic maps for families A00588, A01013 and A01754 was to facilitate QTL identification for resistance to WGR in the three families. The three families used here were selected because their mother trees exhibited resistance to WGR, and the half-sib progenies exhibited a great range of resistance to WGR. Mapping the segregating RAPD markers in such populations will increase the possibility of identification of quantitative trait loci for resistance to WGR. The three genetic maps we report here contain 225, 172 and 234 RAPD markers, respectively. The corresponding average distances between two adjacent markers are 15.63, 20.33 and 13.98 cM. Overall, no serious clustering of RAPD markers were observed and the RAPD markers were evenly spaced along the genomes. With this level of map density, we expect identification of QTLs for commercially importance traits in lodgepole pine should be possible. As well, the availability of a large number of RAPD markers in our maps allows the choice of appropriate RAPD markers for population and evolutionary studies of the species.

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Table 4-1. Putative common markers in three lodgepole pine families

FamilyA00588		FamilyA01013		FamilyA01754	
OAzb--1495 [n]	Uttt--800*	OAzb--1495 [n]	Ufse--310**	OAzb--850 [n]	Utwr--1720*
OAee--1115**	Uttt--845*	OAzb--850	Ufse--430*	OAee--1115**	Utwr--625*
OBzb--1230*	Uttt--960*	OAee--1115**	Ufbr--905*	OGer--1085*	Url--590*
OBzb--750	Uttl--1130*	OGer--1085*	Ufjf--960*	OGer--790*	Utjb--520 [m]
OGer--790*	Utts--1305 [m]	OPAez--1130**	Ufjw--1550*	OPAez--1130**	Ufft--1920**
OPAez--1130**	Utw--1420*	OPAez--730**	Uwzf--800 [m]	OPAez--730**	Ufft--1720*
OPAez--730**	Utwr--1260*	Ueee--570*	Uwrs--1415 [m]	OPAez--920 [n]	Ufft--980 [n]
OPAez--920 [n]	Utwr--1720*	Ueel--335 [n]	Ulet--810*	OPAer--2090*	Ufwf--1205*
OPAer--2090*	Utl--590*	Ueel--960	Ulef--2100 [n]	OPAer--770*	Uflz--985*
OPAer--770*	Utjb--520 [m]	Uete--670*	Ulrz--1230*	OBzb--1230*	Ufse--310**
OPBes--960*	Ufrb--1260	Uefl--320*	Ulte--450**	OBzb--750 [n]	Ufsw--820 [n]
Ueee--1375*	Uftw--1270*	Uelj--830 [n]	Ulwe--725*	OPBes--960*	Ufsj--2100 [m]
Ueee--2255*	Ufft--1720*	Uebf--1125 [n]	Ulwj--800 [n]	Ueee--1375*	Ufbr--890*
Ueee--570*	Ufft--1920**	Ueb--565*	Ullr--1080*	Ueee--2255*	Ufbt--1895*
Ueel--960 [n]	Ufwf--1205*	Uejw--990*	Ullt--415	Ueel--335 [n]	Ufjf--960*
Uete--1230 [n]	Uflz--1305*	Uejw--760 [n]	Ullt--705*	Ueel--960 [n]	Ufjw--2680*
Uetw--965 [n]	Ufse--310**	Urfb--690*	Ulsl--830*	Uete--1230	Uwzf--800 [m]
Uefl--320*	Ufse--430*	Urfb--765*	Ulbe--1715*	Uete--670*	Ulef--2100
Uelr--530 [m]	Ufsw--820 [n]	Urws--1335*	Ulbe--395*	Uetw--965	Ulrz--1230*
Uelj--830	Ufsj--2100 [m]	Urws--765 [n]	Ulbl--865 [n]	Uelr--530 [m]	Ulet--450**
Uebf--920*	Ufbr--890*	Urlw--1230 [n]	Usrf--1455**	Ueb--880*	Ult--750*
Ueb--565*	Ufbt--1895*	Urls--610 [n]	Usrf--670*	Uebf--1125	Ulwj--455*
Ueb--880*	Ufbt--905*	Urlb--455*	Usrf--905*	Uebf--920*	Ullr--1080*
Uejz--715*	Ufjf--960 [n]	Urjs--1160 [n]	Usw--1615*	Uejz--715*	Ullt--705*
Uejw--760 [n]	Ufjw--1550*	Utrz--770*	Ujr--1665*	Urfb--1130*	Ulsl--830*
Uejw--990*	Ufjw--2680*	Utrf--1010 [n]		Urfb--765*	Ulbe--1715*
Urfb--1130*	Uwrs--1415 [m]	Uttt--1140*		Urws--765 [n]	Ulbs--865
Urfb--690*	Ulet--810*	Uttt--540*		Urlb--1270*	Uljz--1495*
Urws--1335*	Ulte--450**	Uttt--800*		Urlb--455*	Usrf--1455**
Urlw--1230	Ult--750*	Uttt--960*		Ursz--1430*	Usw--1085*
Urls--610	Ulwe--725*	Uttl--1130*		Ursz--740*	Usw--1615*
Urlb--1270*	Ulwj--455*	Uttl--920*		Ursw--1340*	Ujr--620*
Ursz--1430*	Ulwj--800	Utts--1305 [m]		Urjw--625*	
Ursz--740*	Ullt--415 [n]	Utw--1420*		Urjs--1160	
Ursw--1340*	Ulbe--395*	Utwr--1260*		Utee--1170*	
Urjw--625*	Uljz--1495*	Utwr--625*		Utrz--410*	
Utee--1170*	Usrf--1455**	Ufrb--1260 [n]		Utrf--1010	
Utrz--410*	Usrf--670*	Uftw--1270*		Utrf--1035*	
Utrz--770*	Usrf--905*	Ufft--1920**		Uttt--1060*	
Utrf--1035*	Usw--1085*	Ufft--980 [n]		Uttt--540*	
Uttt--1060*	Ujr--1665*	Uflz--1305*		Uttt--845*	
Uttt--1140*	Ujr--620*	Uflz--985*		Uttl--920*	

[n] Common markers not mapped onto main linkage groups.

** Common markers across the three lodgepole pine families mapped onto the same parallel linkage groups.

* Common markers between two either two of the lodgepole pine families mapped onto the same parallel linkage groups.

[m] Common markers between either two or across three lodgepole pine families mapped onto different parallel linkage groups.

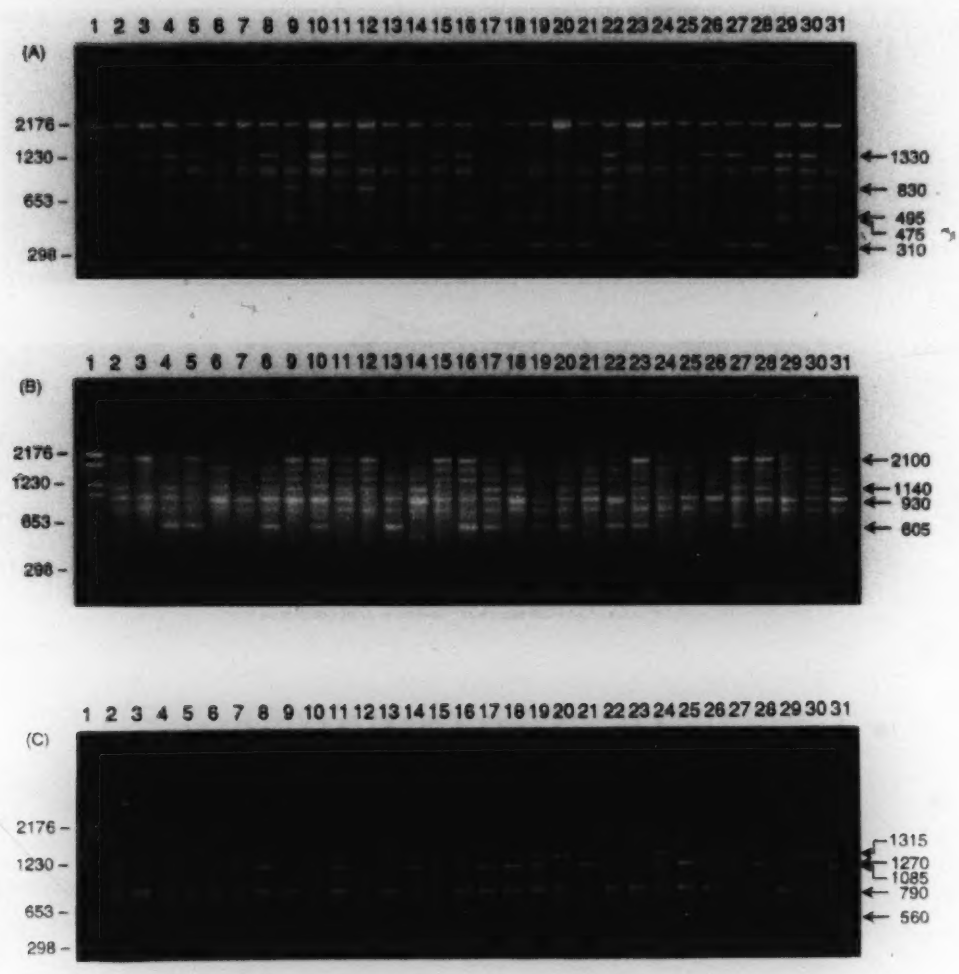


Figure 4-1. RAPD profiles of DNAs from megagametophytes of lodgepole pine half-sib families. Lane 1: DNA molecular weight marker VI (Boehringer Mannheim). Lane 2 -lane 31: RAPDs of family A00588 amplified by primer Uelj (A), RAPDs of family A01013 amplified by primer Ulef (b), and RAPDs of family A01754 amplified by primer OGer (c). The arrows indicate the segregating fragments with their molecular sizes in base pairs on the right.

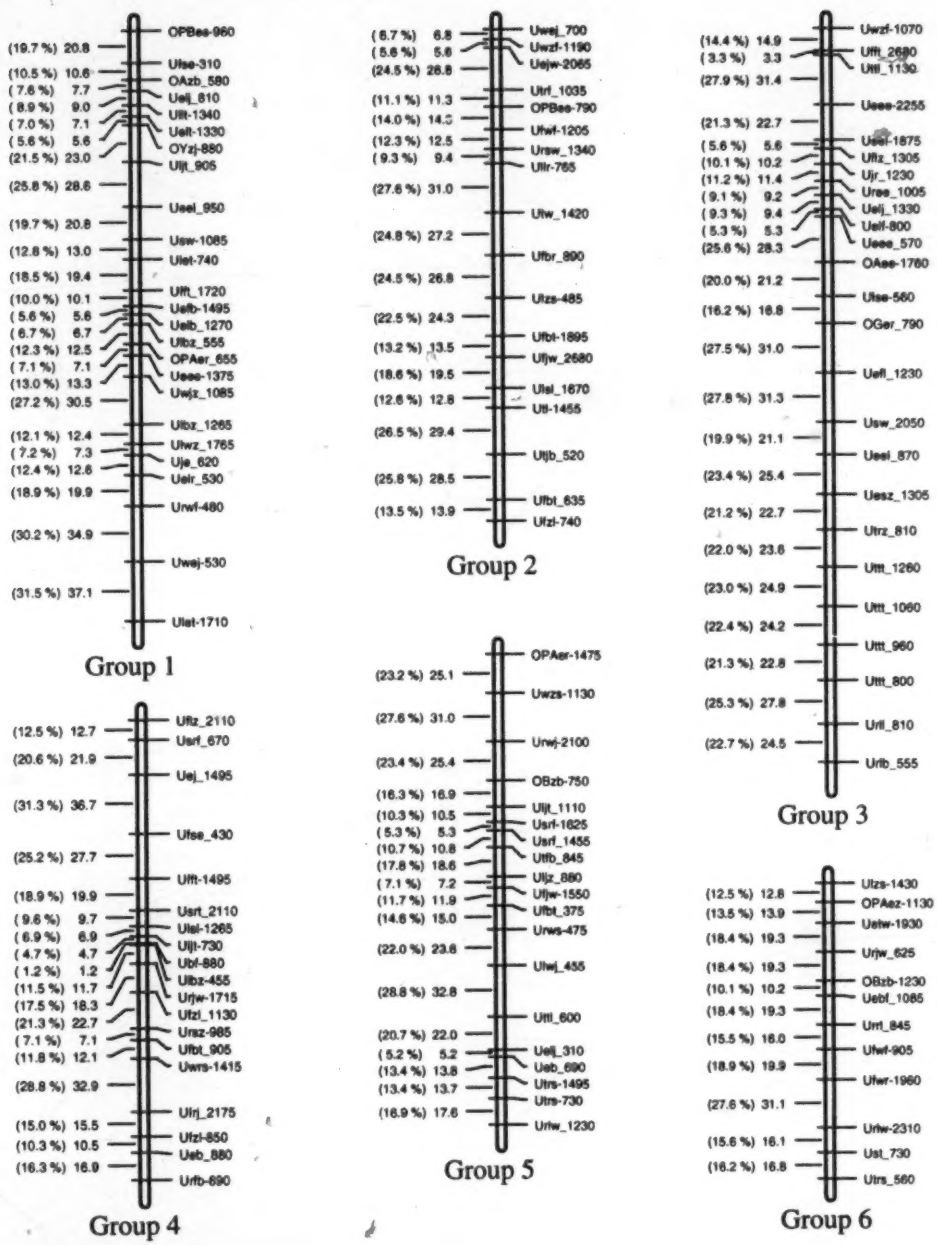


Figure 4-2. Linkage map for lodgepole pine family A00588 based on 225 RAPD markers. Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked.

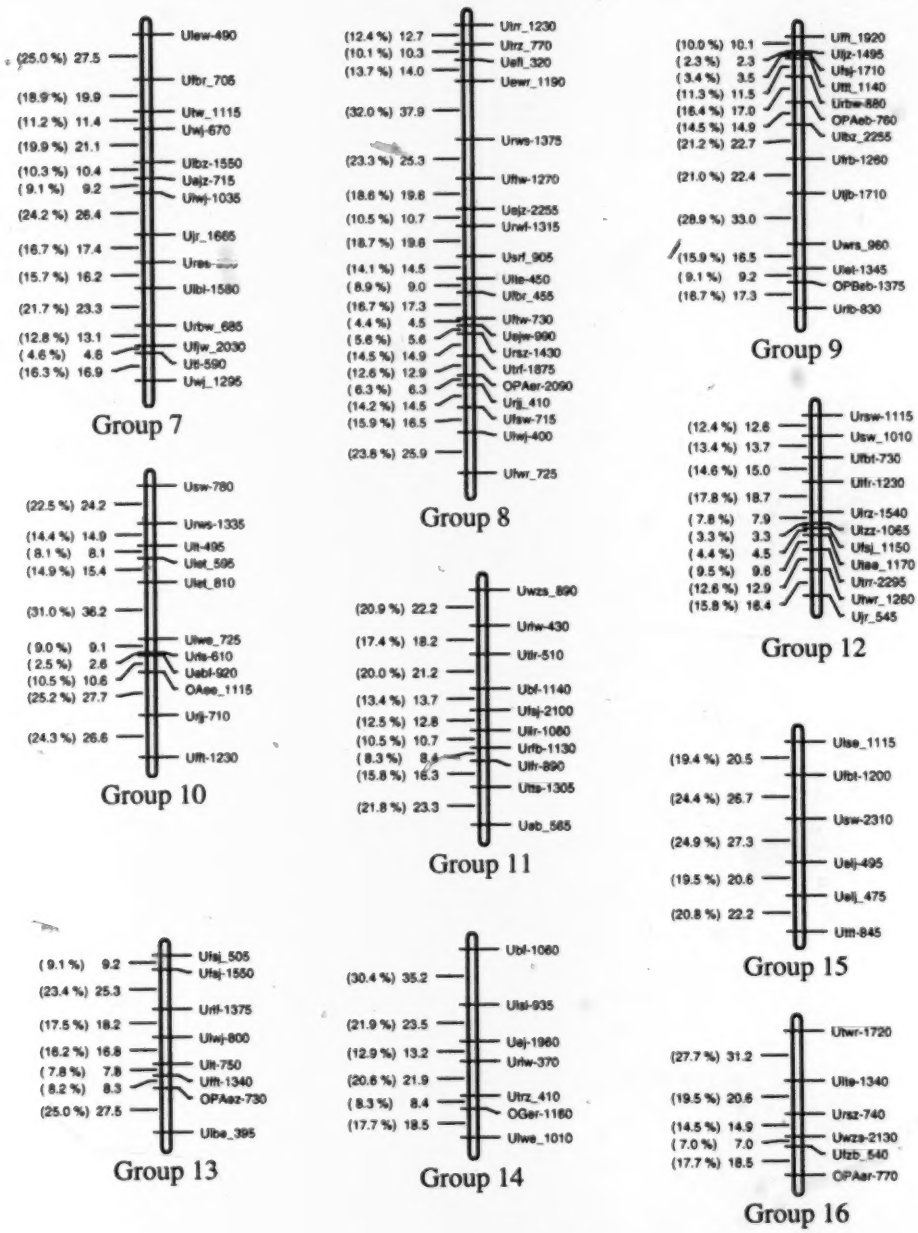


Figure 4-3. Linkage map for lodgepole pine family A00588 based on 225 RAPD marker (continued). Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked.

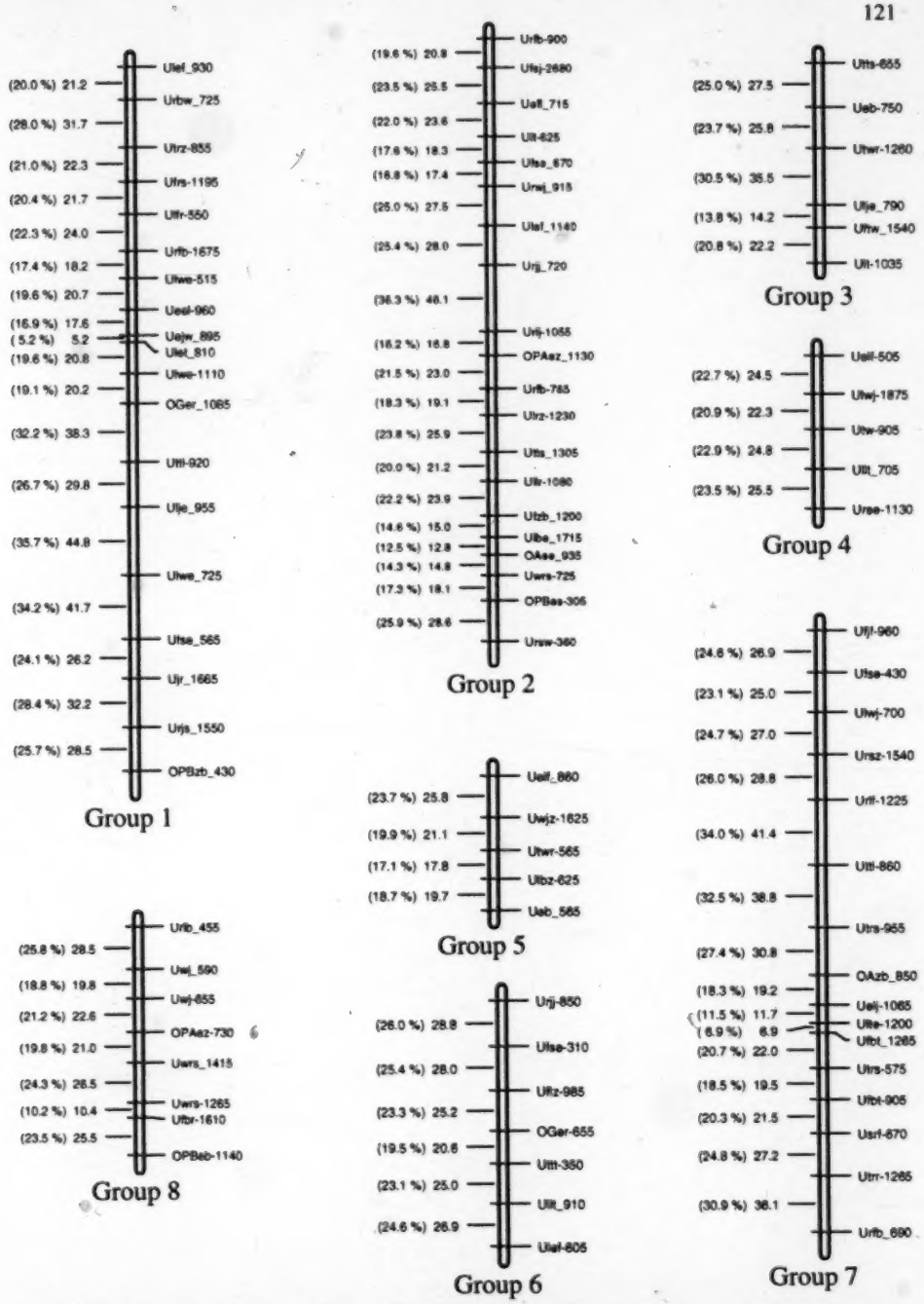


Figure 4-4. Linkage map for lodgepole pine family A01013 based on 172 RAPD markers. Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked.

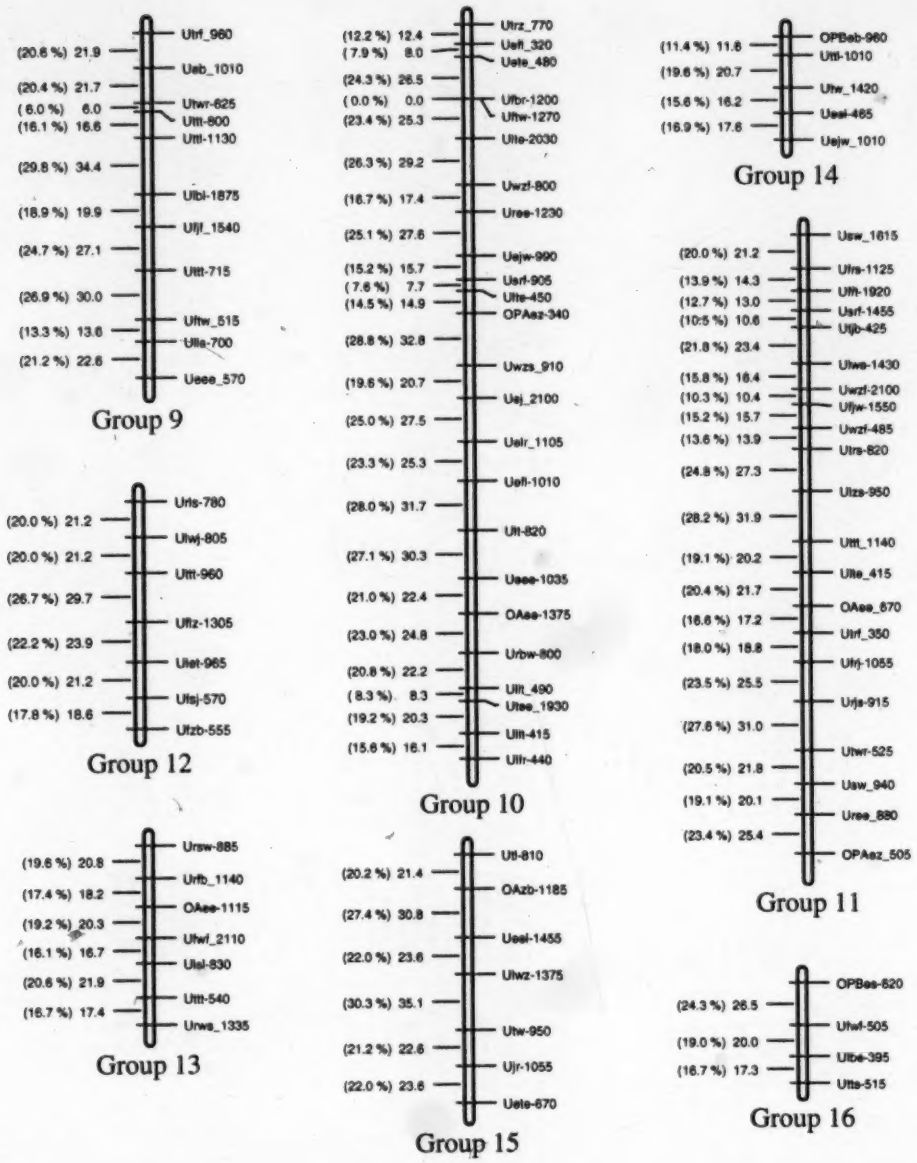


Figure 4-5. Linkage map for lodgepole pine family A01013 based on 172 RAPD markers (continued). Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked.

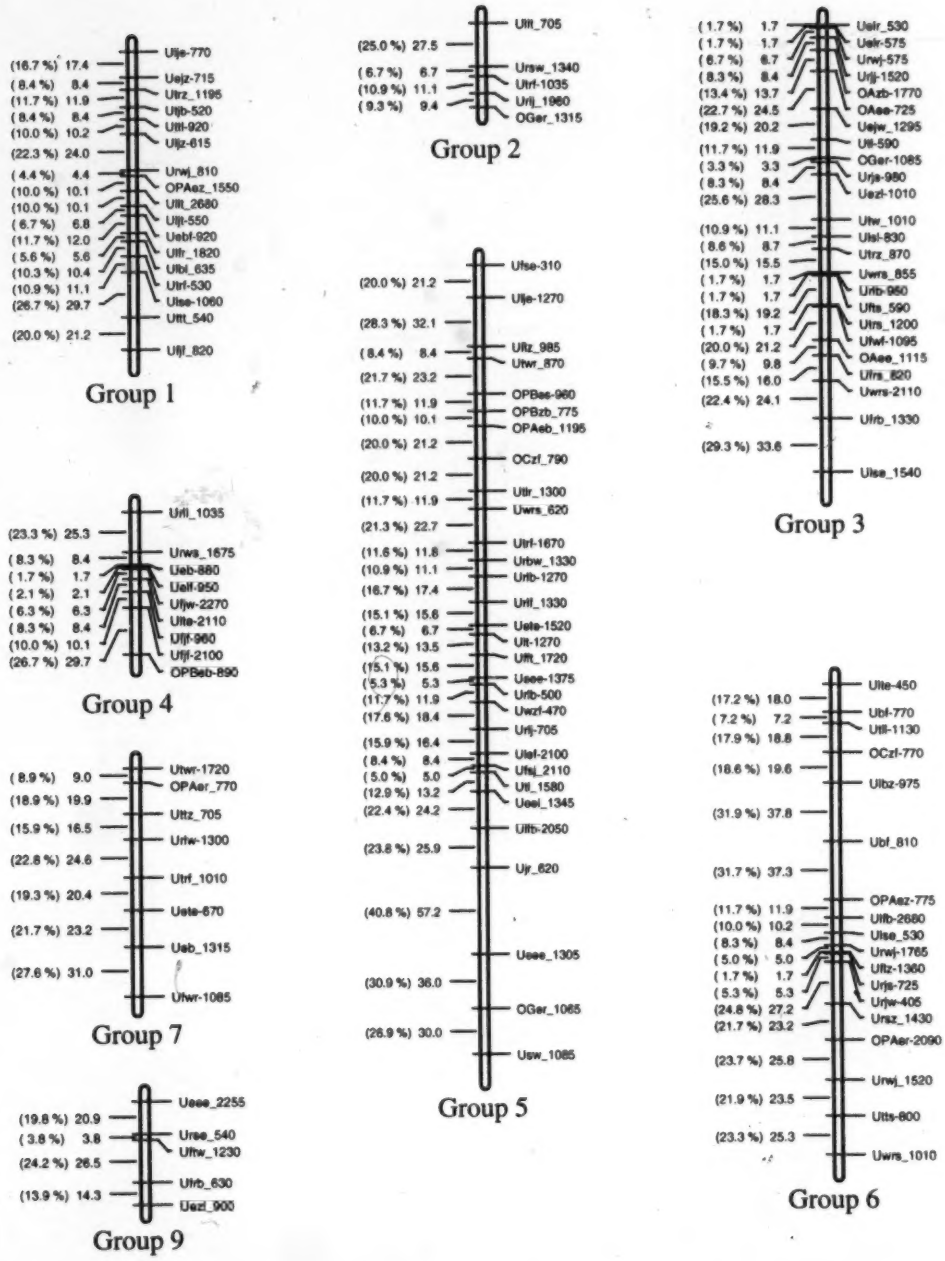


Figure 4-6. Linkage map for lodgepole pine family A01754 based on 243 RAPD markers. Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked.

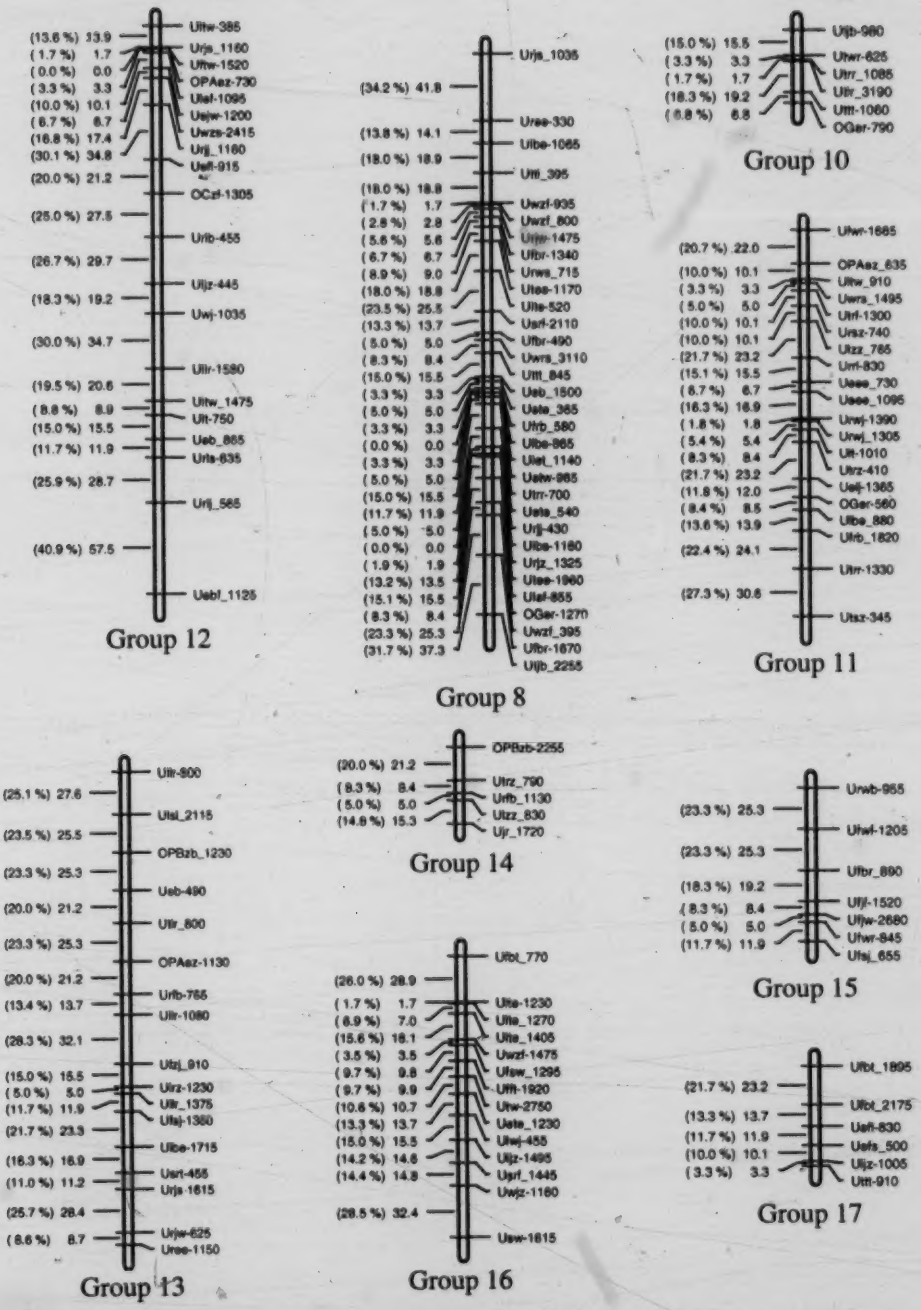


Figure 4-7. Linkage map for lodgepole pine family A01754 based on 243 RAPD markers (continued). Marker names containing the primer ID and fragment size were given to the right of the linkage groups and the recombination frequency (in parenthesis) and Kosambi centimorgan distance were given to the left. The "-" or "_" bar between primer ID and the fragment size of each marker indicated coupling and repulsion phases linked.

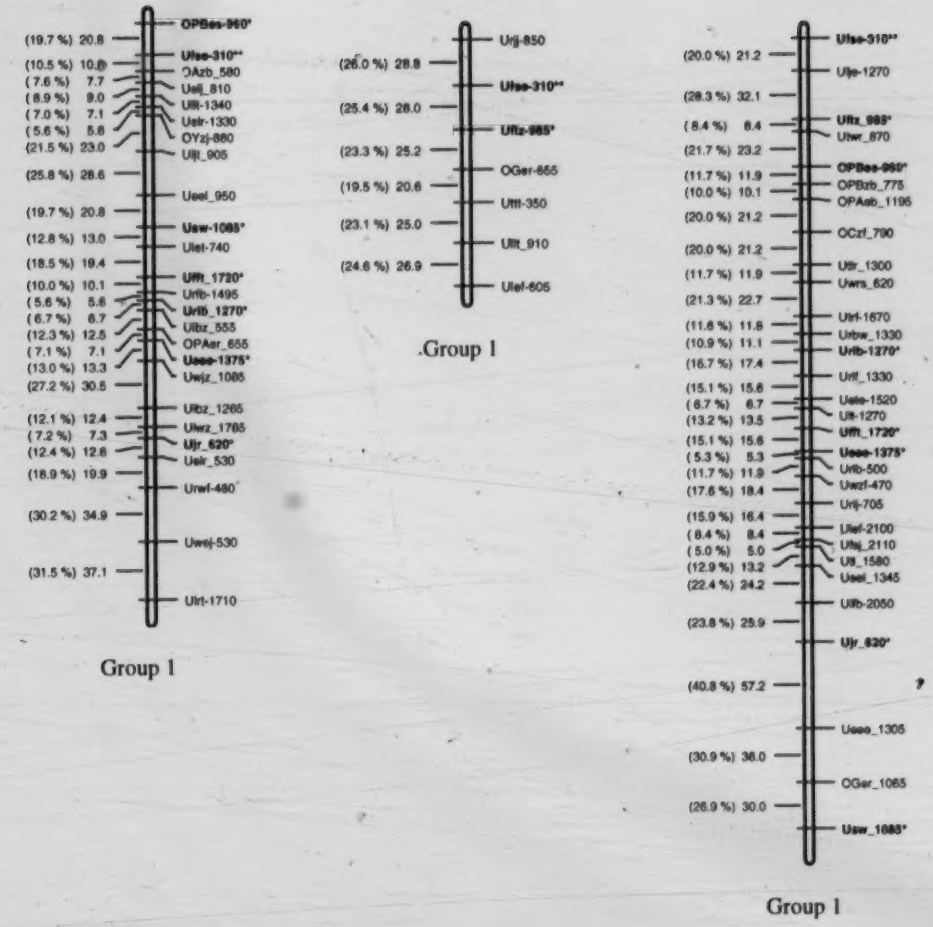
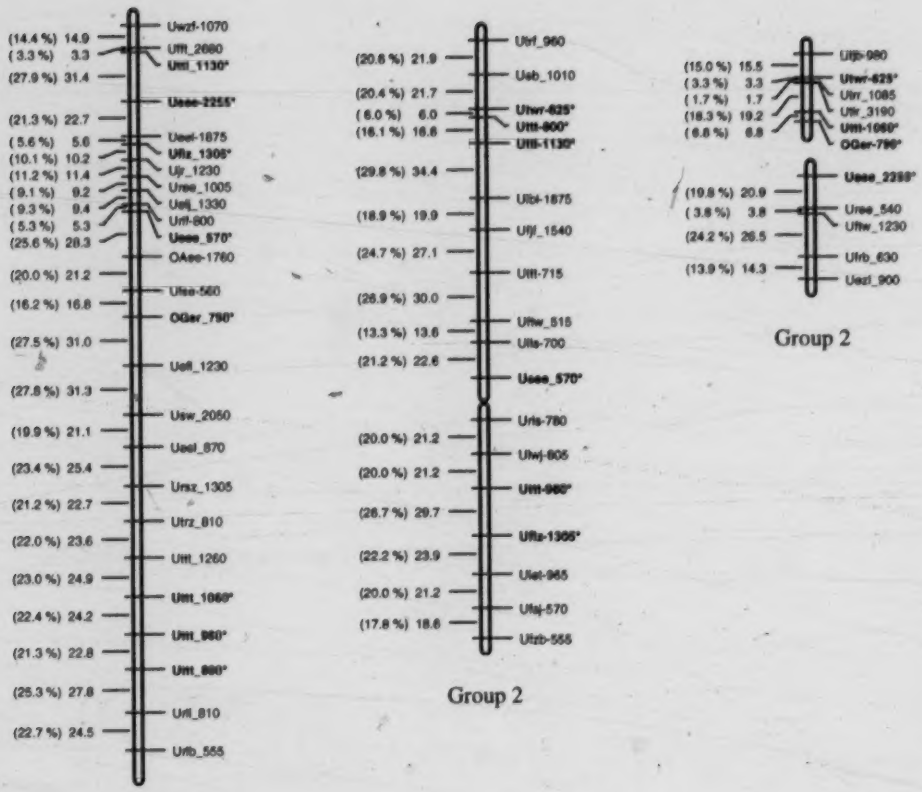


Figure 4-8. Putative parallel linkage group 1 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.



Group 2

Group 2

Group 2

Figure 4-9. Putative parallel linkage group 2 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.

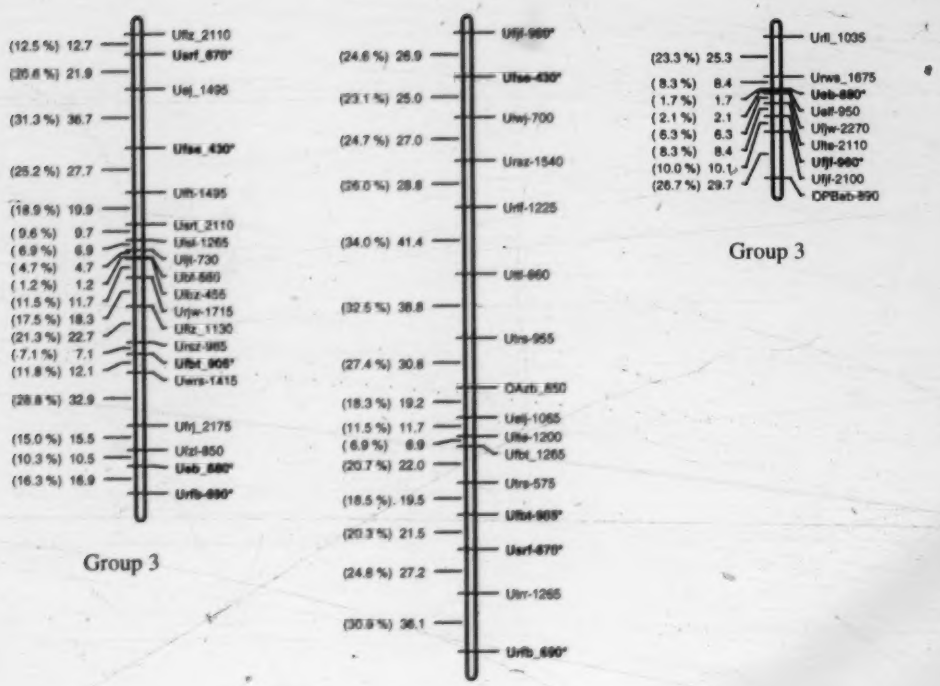


Figure 4-10. Putative parallel linkage group 3 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.

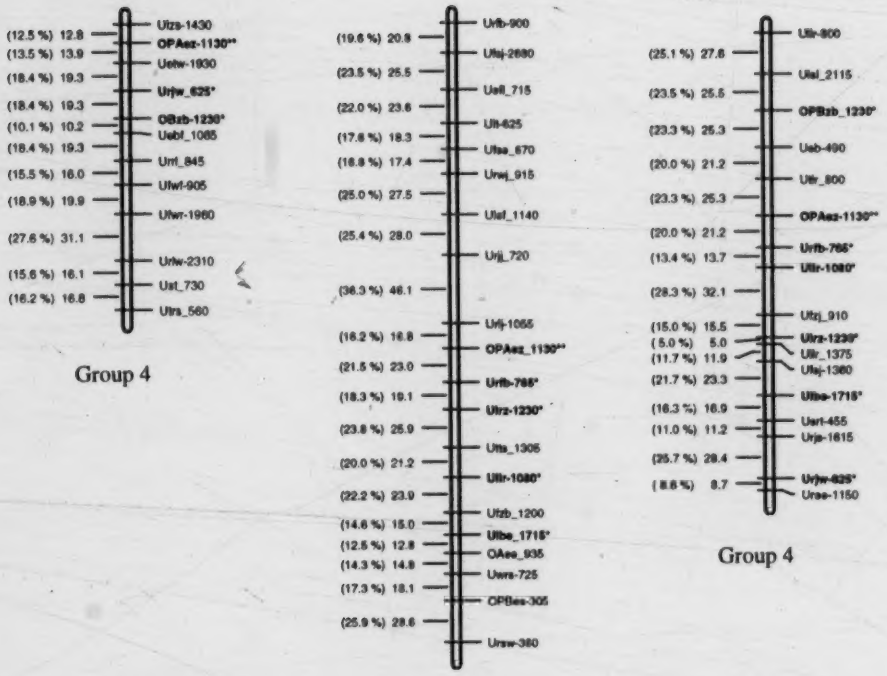


Figure 4-11. Putative parallel linkage group 4 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.

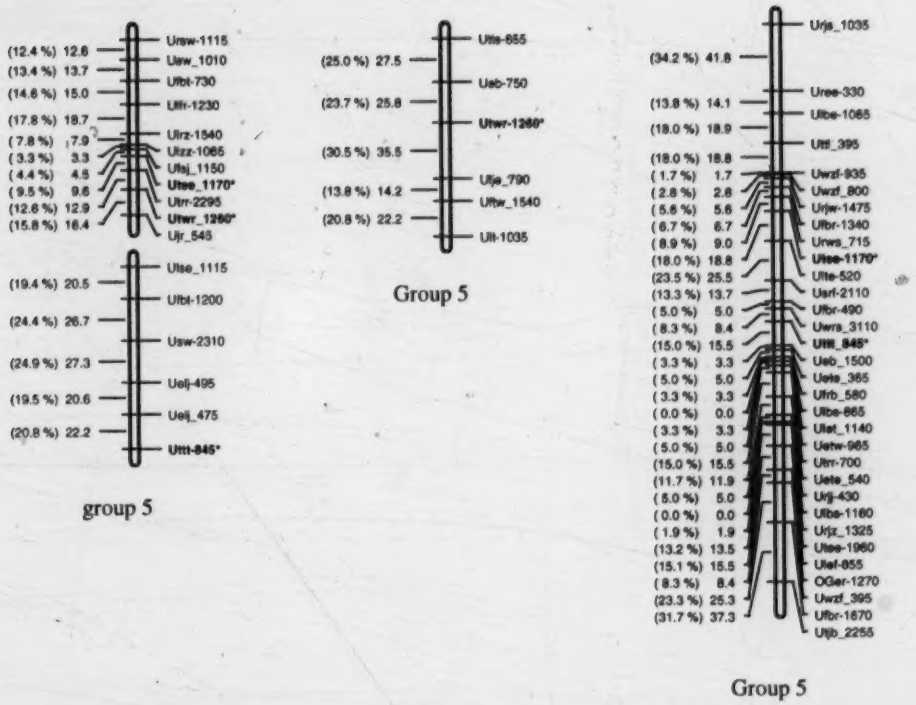
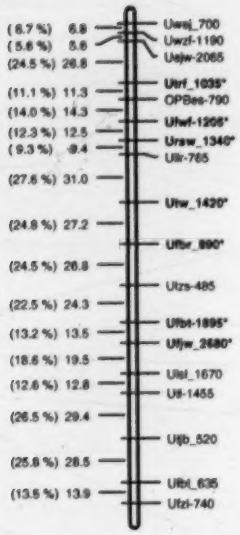
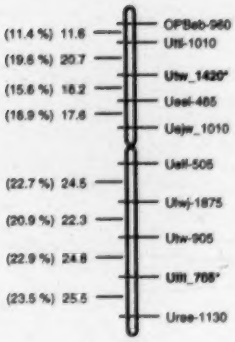


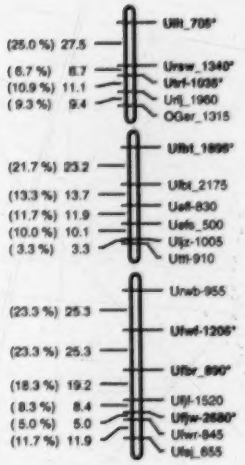
Figure 4-12. Putative parallel linkage group 5 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.



Group 6



Group 6



Group 6

Figure 4-13. Putative parallel linkage group 6 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.

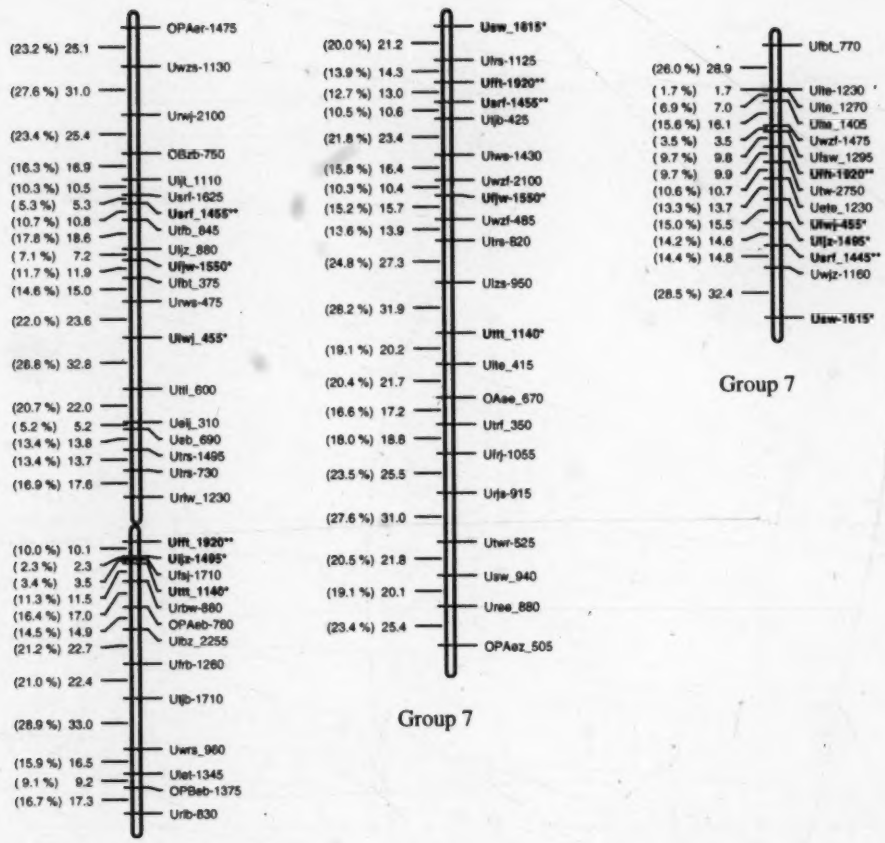


Figure 4-14. Putative parallel linkage group 7 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.

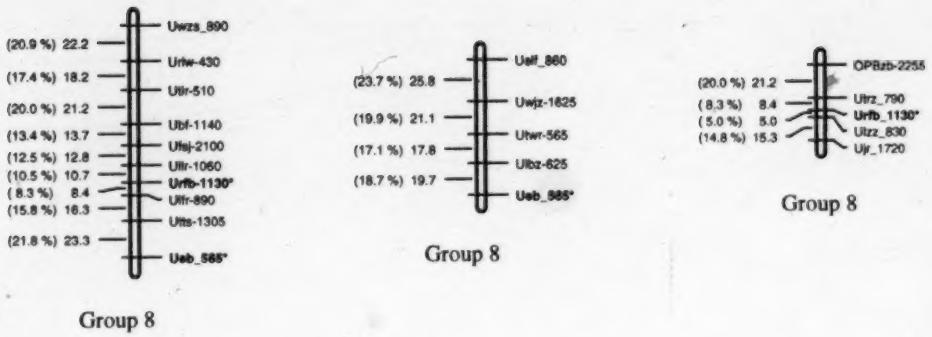


Figure 4-15. Putative parallel linkage group 8 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.

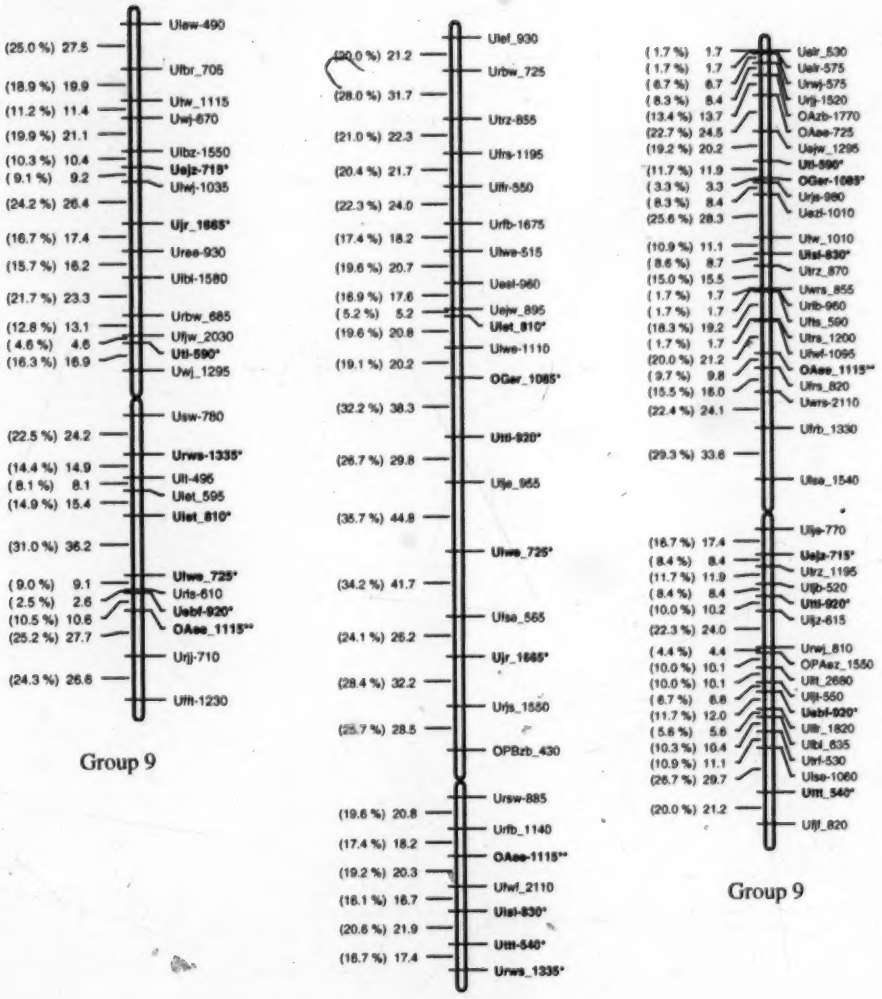
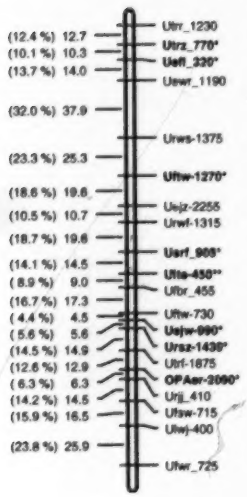
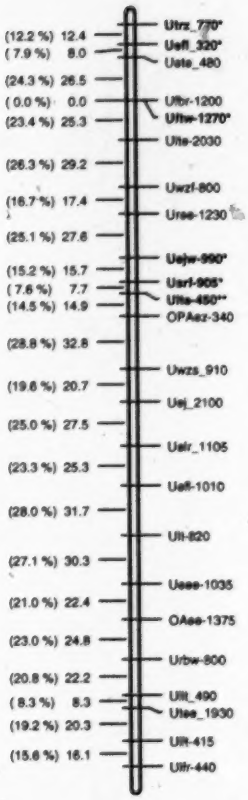


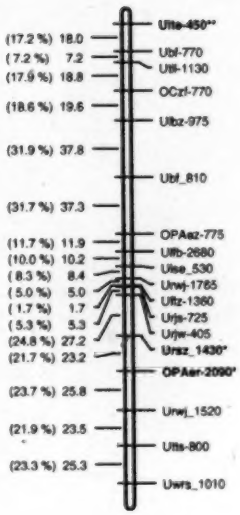
Figure 4-16. Putative parallel linkage group 9 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.



Group 10



group 10



Group 10

Figure 4-17. Putative parallel linkage group 10 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.

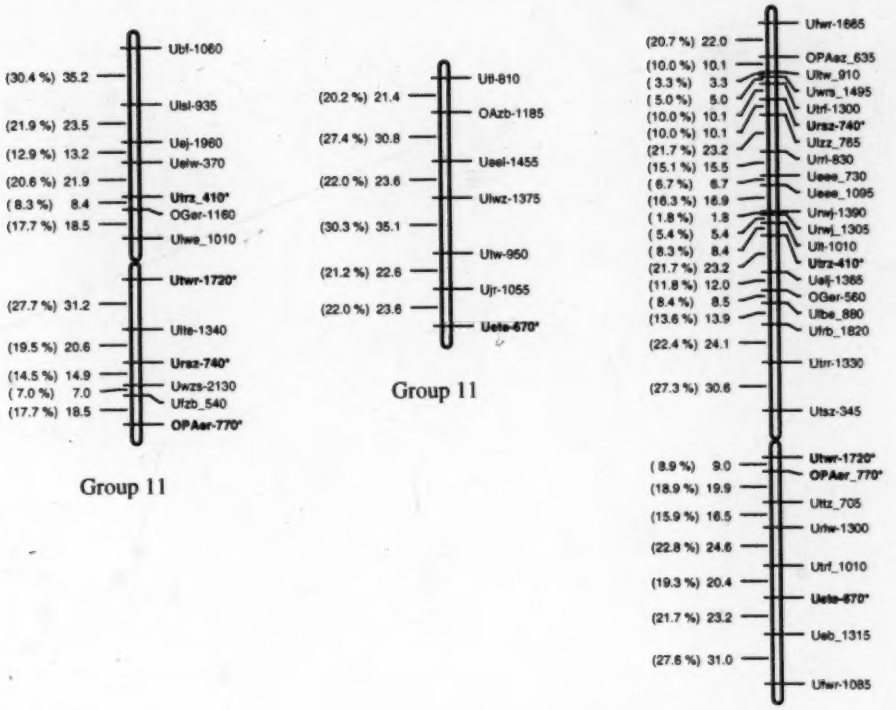


Figure 4-18. Putative parallel linkage group 11 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.

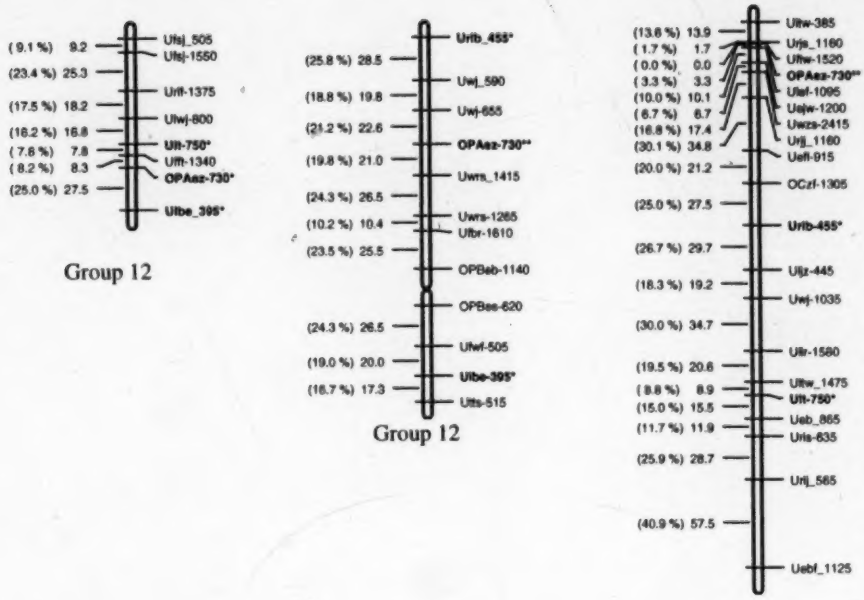


Figure 4-19. Putative parallel linkage group 12 for three lodgepole pine families. Linkage groups of family A00588, family A01013 and family A01754 were displayed from left to the right. "**" indicated common markers across the three families. "*" denoted common markers between either two of the families.

CHAPTER 5 IDENTIFICATION OF QUANTITATIVE TRAIT LOCI FOR RESISTANCE TO WESTERN GALL RUST IN ALBERTA LODGEPOLE PINE

5.1 INTRODUCTION

Many traits in plants and animals exhibit continuous variation in nature. It is believed that such quantitative traits are controlled by multiple segregating genes, modified by environmental effects (Paterson *et al.* 1988). Identification of those genes or quantitative trait loci (QTL) has been an important focus for decades in plant genetics and breeding research.

Early attempts to detect quantitative trait loci for commercially important traits were conducted mostly in crops, by relating the quantitative traits of interest with a few morphological markers (Sax 1923; Thoday 1961; Law 1967), and later with isozyme markers (Tanksley *et al.* 1982; Edwards *et al.* 1987). However, systematic and accurate mapping of QTLs was not possible due to the difficulty in arranging crosses of material that had genetic markers densely spaced throughout the entire genome (Lander and Botstein 1989). Recently, with the development of many types of molecular markers and the advancement of statistical analysis, it is now possible to identify and localize quantitative trait loci of interest on chromosomes based on complete linkage maps (Paterson *et al.* 1988; Stuber *et al.* 1992; Veldboom *et al.* 1994). In the last several years, quantitative trait loci (QTL) for growth traits as well as for disease resistance traits have been successfully identified and mapped in many forest trees using linkage genetic maps (Groover *et al* 1994; Bradshaw and Stettler 1995; Grattapaglia *et al.* 1995; Crouzillat *et al*, 1996; Newcombe and Bradshaw 1996), which will greatly facilitate marker-assisted

selection in tree improvement and probably lead to isolation of genes of interest in the future.

Western gall rust (WGR) is an important fungal disease of lodgepole pine. Damage caused by WGR in lodgepole pine has been reported frequently, especially in young plantations (Powell and Hiratsuka 1973; van der Kamp and Spence 1987; Bella and Navratil 1988; Yanchuk *et al* 1988; Wu *et al.* 1996). Traditionally, efforts to control WGR fungal infection have focused on either containing the fungus (Tsuneda and Hiratsuka 1979; Blenis *et al.* 1988; 1996; Currie 1995) or increasing genetic resistance in the host (Martinsson 1980; Yanchuk *et al.* 1988; Klein 1991). However, as the management of lodgepole pine plantations becomes intensive, the effective manipulation of both fungus and hosts must be considered the prime objective.

Many field investigations and greenhouse trials demonstrate that lodgepole pine shows a remarkable variation in resistance to WGR (Martinsson 1980; Yanchuk *et al.* 1988; van der Kamp 1988b; 1989; Blenis *et al.* 1993; Kojwang and van der Kamp 1991; Wu *et al.* 1996; Yang *et al.* 1997). Resistances in lodgepole pine varied in a continuous fashion when expressed as the number of infections per tree (van der Kamp 1989). In greenhouse studies, non-infection and complete galls as well as partial galls were commonly observed among individuals of lodgepole pine (Yang *et al.* 1997). It seems that polygenic resistance in lodgepole pine predominates, however single major resistance may play a role in the lodgepole pine-western gall rust pathosystem (Kojwang and van der Kamp 1991).

Identification of QTLs for resistance to WGR in lodgepole pine will help understand the genetic basis for resistance in the host and lead to isolation of the resistance genes,

which will greatly improve the management of both the rust and the host. This chapter reports the detection and mapping of QTLs for resistance to WGR in three lodgepole pine families. The QTLs for resistance to WGR were identified and mapped based on the genomic maps constructed using RAPD markers amplified from megagametophyte DNAs of each corresponding seedling of the three lodgepole pine half-sib families.

5.2 MATERIALS AND METHODS

5.2.1 Plant materials

Seeds of three open-pollinated lodgepole pine families, family A00588, family A01013 and family A01074 were collected in a seed orchard in Alberta. These three families were chosen because their maternal trees were disease free to WGR in natural stands and their half-sibs showed a large phenotypic variance in resistance to WGR in long-term field and greenhouse screening. The three lodgepole pine maternal trees originated from Alberta. Tree A00588 was 74 years of age at the time of seed collection in 1976 and near Wolfcreek, at 1036 m elevation, latitude $54^0$36' N and longitude $119^0$03' W. Tree A01013 was near Judy Creek, at 1070 m elevation, latitude $54^0$26' N and longitude 115^0 34'W. It was 133 years of age at the time of seed collection in 1977. Tree A01754 was 77 years of age at the time of seed collection in 1978 and near Windfall, at 1000 m elevation, latitude $54^0$01' N and longitude 116^0 34'W.

Seeds of the three families were germinated in the greenhouse in plastic trays (Ventblock ® 45, Beaver plastic Ltd.) used for growing the seedlings. Each tray contained 45 cavities (350 cm^2/cavity). Each cavity was filled with peat moss adjusted to

pH 5-5.5 by the addition of dolomite lime. For a 113-L bale of peat moss, 358 g of lime was added. The medium was saturated with enough water to last until after sowing.

Seeds were directly sown into the cavities on May 9, 1996 with 2 seeds per cavity. For the first two weeks after sowing, deionized water was applied daily to maintain soil moisture. After two weeks, each cavity had 1-2 germinations, and the seedlings were randomly thinned to one plant per cavity. The megagametophytes of the remaining seedlings were then obtained by their removal from the extending cotyledonary needles of the germinating seedlings prior to natural abscission. The megagametophytes were stored at -20 ^{0}C and used for DNA extraction. Their corresponding seedlings were kept growing in the greenhouse under a natural photoperiod with controlled temperature of 20-25 ^{0}C. A solution of complete fertilizer 20-20-20 (200 ppm) was also applied at two-week intervals in conjunction with seedling watering.

5.2.2 Artificial inoculation and resistance evaluation

Seven weeks after germination, the seedlings were inoculated with two single-gall spore sources, which were genetically different based on their RAPD analyses. Half of the seedlings were inoculated with one single-gall isolate collected from Alberta, representing the spore source from lodgepole pine. The other half were inoculated with the second single-gall isolate collected from Manitoba, representing the spore source from jack pine.

The rust spore viability was tested on 0.2% agar on a microscope slide at 25^{0}C before inoculation. The germination rates for both spore sources were above 85% based on estimation under a microscope. The inoculation procedures followed the torn needle

method developed by Myrholm and Hiratsuka (1993). Six months after inoculation, disease infections were evaluated based on the evaluation criteria developed at the Northern Forestry Centre, Canada (Klein *et al.* 1991). A rating of 0 represents a lack of symptoms, whereas a rating of 5 represents a globose gall. Ratings of 1, 2, 3 or 4 were given to seedlings according to their gall appearances, from a slight sign of infection to partial gall.

5.2.3 Genomic map construction

Genetic linkage maps were constructed for each family. DNAs from ninety megagametophytes of family A00588 were amplified with each of 110 pre-selected primers. DNAs of family A01013 from 60 megagametophytes were amplified with the 96 selected primers. Extracted DNAs from another set of 60 megagametophytes of family A01754 were also amplified with the 104 chosen primers. The primers were selected based on their abilities and consistencies in generating segregating markers in each family after screening 840 random primers.

Segregating markers showing a Mendilian 1:1 ratio were identified for each family and used for their map constructions. Map construction was performed with Mapmarker for Macintosh V2.0. The markers were assigned into possible linkage groups with an LOD value of at least 3.0 and a maximum recombination of 0.35. The map orders were determined using two-point and multipoint analysis. The details of map construction for the three lodgepole pine families were reported in Chapter 4. Sixteen main linkage groups were identified for family A00588. The sixteen linkage groups contained 225 RAPD markers covering a total distance of 3517.5 cM. In family A01013, one hundred and

seventy two RAPD markers were mapped onto 16 main linkage groups covering a total distance of 3496.0 cM. The linkage map for family A01754 was represented by 17 main linkage groups with 234 RAPD markers spanning a total distance of 3398.2 cM. Quantitative trait loci analysis for resistance to WGR was performed based on the above linkage groups of each family.

5.2.4 QTL identification

QTL analysis was performed for each family against the two spore sources by interval mapping implemented by MAPMAKER-QTL under the backcross model (Lander and Botstein 1989). The disease score of the seedlings corresponding to the megagametophytes used in map construction were recorded and transformed by log(1+score) from their disease score values. An LOD threshold of 3.20, 3.15 and 3.25 was chosen respectively for family A00588, A01013 and 01754 to achieve an overall false positive rate less than 0.05 in declaring the putative QTLs for each individual test. The LOD thresholds of the three families were chosen according to the formula $1/2(\log_{10} e)(Z_{a/M})^2$ (Lander and Botstein 1989) based on their number of marker intervals scanned for QTLs, M, and a maximum false positive rate of 0.05. $Z_{a/M}$ was defined as the number of standard deviations beyond which the normal curve contains probability a/M. For each LOD peak, a 1.0 LOD support interval was used to determine the left and right boundaries. The percentage of variance explained by each single QTL as well as by the multiple QTL model as estimated by interval mapping was also reported.

5.3 RESULTS

5.3.1 QTLs in family A00588

The results of identification of QTLs in family A00588 for resistance to WGR are summarized in Table 5-1 and the positions in the linkage maps depicted in Figure 5-1. Three putative QTLs, QTLI, QTLII, and QTL III were detected at a LOD score of at least 3.20 or above. Among them two putative QTLs, QTLI and QTLII are related to the resistance to WGR from the Alberta source. The two QTLs are located in linkage group 9. The first one is between marker Ufsj-1710 and marker Uttt_1140 with the most likely position at marker Ufsj-1710. This QTL explains 35.6% the total phenotypic variance in resistance with an effect of decreasing the disease value at 0.418. The second one is located between marker Ulbz_2255 and marker Ufrb-1260 with the most likely position at 16 cM away from marker Ulbz-2255. This QTL explains 32.8% of the total phenotypic variance in resistance and has an effect of reducing the disease value at 0.408. The two QTLs jointly explain 40.2% of the total phenotypic variance in resistance in a two-QTL model.

The third putative QTL, QTLIII, identified in family A00588 is also located in linkage group 9, between marker Ufft_1920 and marker Uljz-1425 with the most likely position at 2.0 cM away from marker Ufft_1920. This QTL is associated with resistance to WGR from the Manitoba source and explains 23.3% of the total phenotypic variance in resistance with effect of reducing the disease value of 0.427.

It is observed that the putative QTL related to the resistance to WGR from the Manitoba source is located close to the two QTLs for resistance to the Alberta rust source. The support interval for QTLIII overlaps to some extent to that of QTLI and

QTLII. The likely position of the QTLIII, which is for the Manitoba source resistance, is about 8.1 cM away from that of QTL I, the QTL for resistance to the Alberta rust source.

5.3.2 QTLs in family A01013

Table 5-2 summarizes the results of detection of QTLs for resistance to WGR in family A01013. In total, nine putative QTLs were identified. Five of them, QTLI, QTLII, QTLIII, QTLIV and QTLV, are associated with resistance to the WGR from Alberta source and the other four, QTLVI, QTLVII, QTLVIII and QTLIX, to the WGR from the Manitoba source. The nine QTLs are distributed in six linkage groups, 1, 7, 10, 11, 13 and 16. Among them, four QTLs, QTLIII, QTLIV, QTLVI, and QTLVII are located in linkage group 10, two of which are related to the resistance to the rust from the Alberta source and the other two to the rust from the Manitoba source. The positions of these QTLs in these linkage groups are presented in Figure 5-2.

The QTLs identified in family A01013 have a great effect on the resistance to WGR. Each QTL explains about 81.7% to 86.5% of the total phenotypic variance in resistance with effects of decreasing the disease value between 0.612 to 0.726. The most likely positions of the nine QTLs are about 10 to 34 cM away from its mostleft markers. The five-QTL model explains 87.4% of the total phenotypic variance for resistance to WGR from the Alberta source while the four-QTL model explains 84.2% of the total phenotypic variance for resistance to the rust from the Manitoba source.

One QTL for resistance to the Manitoba source in linkage group 10 (QTLVII), was found close to one of the QTLs for resistance to the Alberta source in the same linkage

group (QTL IV). The support intervals for these two QTLs overlap to some extent. The most likely positions for these two QTLs are about 4.0 cM apart.

5.3.3 QTLs in family A01754

The results of identification of QTLs for resistance to WGR in family A01754 were presented in Table 5-3. Two putative QTLs were detected in this family, one for resistance to the rust from the Alberta source (QTL I) and another (QTLII) for resistance to the rust from the Manitoba source. The putative QTL for resistance to the rust from the Alberta source is in linkage group 13 and the most likely position is 12.0 cM away from marker Ufsj-1360 (Figure 5-3). Another QTL for resistance to the rust from the Manitoba source was detected in linkage group 3 (Figure 5-3). The most likely position of this QTL is 26 cM away from marker Ufrb_1330.

Both of the QTLs explains most of the total phenotypic variance in resistance at a proportion of 87.3 and 81.5% respectively. The effects of the two QTLs on the disease value are 0.709 less for resistance to the rust from the Alberta source and 0.650 less for the resistance to the rust from the Manitoba source.

5.3.4 QTL associations among families

The QTL associations among families were inferred based on their putative parallel linkage groups. One QTL for resistance to the Alberta source in family A01013 (QTLV) in linkage group 11 was found in the same parallel linkage group (parallel linkage group 7) as all three QTLs in family A00588. The two families share several common markers in parallel linkage group 7. Marker Uttt_1140, however, was found to be common within

the QTL support intervals for both QTL V in family A01013 and two QTLs (QTL I and QTL III) in family A00588. The QTL for resistance to the Manitoba source in family A01754 (QTLII) and two QTLs (QTL I and QTL VIII) in family A01013 are located in parallel linkage group 9. However, no common markers were found within their support intervals.

5.4 DISCUSSION

QTL identification using half-sib family pedigrees has been successfully demonstrated in domestic animals for the growth and production traits (Beever *et al* 1990; Georges *et al* 1995), as well as in *Eucalptus* for the growth and wood quality traits (Grattapaglia *et al* 1996). This study was designed to identify QTLs for resistance to WGR in lodgepole pine using open-pollinated families.

The special feature of half-sib families in conifers is that the maternal tree contributes to the haploid megagametophytes as well as to the embryos. Thus, heterozygous loci in the maternal tree will segregate in a 1:1 ratio among the haploid megagametophytes of its seeds. Half of the genetic constitution in a diploid embryo is contributed by the maternal tree, and will be identical to that of the corresponding megagametophyte. Thus, the identification of quantitative trait loci can be made by relating the genetic markers generated from the megagametophytes to the quantitative traits of the corresponding embryos.

In open-pollinated family analysis, only segregating loci are informative and can be used in map construction and QTL identification. Selection of the family for QTL analysis is very important. For any QTLs to be detected using the open-pollinated family

analysis by relating genetic markers to the phenotypes of half-sibs, two basic conditions must be met. The first one is that the quantitative trait loci have to be heterozygous in the maternal tree. The second condition is that there is also a heterozygous genetic marker linked to the QTL. In this study, three families were selected from the maternal trees with good resistance to WGR and their half-sib families exhibited great variation in resistance to the disease based on field and greenhouse observations. This indicated that the QTLs for resistance to WGR might be segregating among the half-sibs. The average number of genetic markers used for QTL identification for the three lodgepole pine families is 210, which has a reasonable probability of detecting the genetic markers linked to the QTL.

QTL mapping was performed by interval mapping implemented by MAPMAKER-QTL (Lander and Botstein 1989) under a backcross model. The MAPMAKER-QTL program maximizes the chance of detecting the QTLs since the program allows the use of flankink markers. However, this procedure still has the possibility of detecting positive false QTLs. Usually, the possibility of detecting a positive false QTL increases as the number of marker intervals scanned along the genome increases at a given LOD score. Lander and Botstein (1989) suggested appropriate LOD scores for different sizes of genomes. We have chosen LOD score thresholds of 3.20, 3.15 and 3.25 for family A00588, family A01013, and family A01754 respectively to avoid an overall false positive rate less than 0.05. With these LOD values, we have identified 14 QTLs for the three families. Most of the LOD scores are higher than the thresholds.

The required number of progeny for detection of a QTL depends on the effect of the QTL. The larger the effect of the QTL, the less the number of progeny that are needed. However, compared to traditional approaches for mapping quantitative trait loci,

the interval mapping method increases the power and reduces the sample size required for detection of QTLs with similar effects. Lander and Botstein (1989) showed that for a linkage map with markers every 20 cM throughout the genome, interval mapping would need 16% less than the required number of progeny for QTL detection.

Selective genotyping of the progeny at the extremes of the phenotypic distribution can also increase the efficiency of QTL identification and thus reduce the required number of progeny that must be genotyped to detect a QTL. In general, interval mapping using a 20 cM linkage map combined with selective genotyping of the individuals located in the 5% tails of the distribution could lead to a reduction of up to 7-fold in the number of required progeny compared to traditional QTL detection approaches. According to the estimation of required progeny size by Lander and Botstein (1989), if a QTL explained 15% of total phenotypic variance, then in order to be detected at a 50% chance in a backcross design, about 110 progeny must be genotyped using the traditional approach of QTL detection. However if the interval mapping method is used, together with a selective genotyping strategy, the number of progeny required for detection of the QTL is reduced to about 25.

QTLs with large effects have been reported in many studies for disease resistance traits as well as for growth traits (Paterson *et al.* 1988; Chen *et al.* 1994; Newcombe and Bradshaw 1996), which support the idea of a few major genes controlling large proportions of the total variation in a wide range of quantitatively inherited traits (Grattapaglia *et al.* 1995). The QTLs detected in this study explained the total phenotypic variance in resistance from a range of 23.2% to 87.3%. The QTLs that accounted for a higher proportion of the total phenotypic variance suggest major resistance genes exist in some of the lodgepole pine families while the QTLs controlling smaller proportions of

the total phenotypic variance might reflect a minor effect of other resistance genes. The number of QTLs and their effects identified in the three lodgepole pine families seemed to conform to their resistance performances in the greenhouse screening, which have been reported in Chapter 3. Among the three families, family A01013 was more resistant to both the WGR source from Alberta and the Manitoba source, and its resistance scores tended to have more non-infections. Consequently, five and four QTLs were detected for resistance to the Alberta and the Manitoba sources of WGR respectively, each with a major effect. Family A00588 was less resistant to WGR, with more complete and partial galls, especially from the Manitoba source. Two QTLs were identified in this family for resistance to the rust from the Alberta source, explaining 40.2% of the total phenotypic variance in resistance and one QTL was detected for resistance to WGR from the Manitoba source, controling only 23.2 % of the total phenotypic variance in resistance. In family A01754, one QTL was identified for resistance to WGR from the Alberta source and another QTL for resistance to the rust from Manitoba. Both QTLs demonstrated a large effect, each accounting for more than 80% of the total phenotypic variance in resistance. It is expected in this family that the distributions of the disease scores among the half-sibs tend to have more either non-infections or complete galls, considering the segregation of one QTL with a major effect among its half-sibs.

Major gene resistance to WGR has been reported in Scots pine (van der Kamp 1991a). In lodgepole pine it has been proposed that a single major resistant gene may play a role in the pathosystem between the rust and the host within a predominately polygenic system (Kojwang and van der Kamp 1991). In this study, eleven of fourteen QTLs

showed major effects. Among them are nine QTLs detected in family A01013, suggesting an accumulation of resistant genes in this family.

Clustering of disease resistance genes has been reported in crops. In potato (*Solanum tuberosum*), loci of two genes for resistance to potato virus X were located closely with loci controlling resistance to late blight disease (*Phytophthora infestans*) (Gebhardt 1994). The associations among QTLs observed in this study might also suggest the clustering of some WGR resistance genes. In family A00588 two QTLs, one for resistance to WGR from the Alberta source (QTLI) and one for resistance to the Manitoba source (QTLIII), were close to each other in the same linkage group. The most likely positions of the two QTLs were about 8.1 cM apart. A similar association was found for another two QTLs in family A01013, QTLIV and QTLVII. QTLIV was related to resistance to WGR from the Alberta source while QTLVII was related to resistance to the rust from the Manitoba source. The two QTLs were mapped in linkage group 10 with their most likely positions being 4.0 cM apart. However, it still difficult to tell whether the two QTLs are the same gene without further analysis, considering a distance of 1cM could be 4000 kilobases or more in conifers (Neale and Williams 1991). The possible associations of the QTLs among the three families were also examined in this study. It seems that QTLV in family A01013 showed some relationships with two QTLs in family A00588 (QTLI and QTLIII). QTLII in family A01754 and two QTLs in family A01013 (QTLI and QTLVIII) were likely in the same parallel linkage groups. Further studies are needed to test the possibility of a common identity of these QTLs among families.

The reliability of QTLs is one of the major concerns in QTL identification. In this study, the LOD scores of the 14 QTLs ranged from 3.86 to 9.37, corresponding to an

overall false positive rate of 0.012 to 1.7×10^{-7} respectively. The false positive rate implies the possibility of a spurious QTL even though the QTL is detected at a significance level. Lower false positive rates give a higher credibility of the QTL. Given the fact that the progeny size was 30 and 45, we anticipate the inclusion of spurious QTLs, especially those with LOD score below 4.90 (corresponding to a false positive rate of approximately 1×10^{-3}). Five QTLs are in this category, QTLIII (LOD=3.86) in family A00588; QTLI (LOD=4.04), QTLVI (LOD=4.25) and QTLIX (LOD=4.41) in family A01013; and QTLII (LOD=3.26) in family A01754. The other nine QTLs have LOD scores that ranged from 4.96 to 9.37 and should not be considered as spurious QTLs. This is especially true for the QTLs with LOD score greater than 6.70 (corresponding to a false positive rate of 1×10^{-4}). They include QTLI (LOD=8.30) in family A00588; QTLV (LOD=6.95), QTLII (LOD=8.05) and QTLIV (LOD=9.37) in family A01013.

Marker-assisted selection (MAS) is considered an effective complement to traditional selection practices in plant breeding programs. It provides a potential for increasing selection efficiency by allowing earlier selection and reducing the plant population sizes that are used during selection (Staub *et al.* 1996). It is also advantageous when the phenotypic selection is difficult due to a low heritability of the character of interest or when phenotypic selection is expensive because the evaluation of the character is costly.

Marker-assisted selection can be initialized only after a strong association between markers and the traits of interest is identified. The identification of such associations is primarily based on the exploration of the linkage disequilibrium between the genetic markers and the quantitative trait loci. MAS can be used as long as the linkage

disequilibrium is maintained. However, the degree of linkage between the genetic markers and the quantitative trait loci affects the efficiency of MAS. The effectiveness of MAS decreases as the linkage distance between the markers and the QTL increases (Staub *et al.* 1996). Random mating during breeding will reduce the linkage disequilibrium more rapidly for QTLs loosely linked to markers than for tightly linked loci (Dudly 1993).

QTLs identified using open-pollinated family analyses reflect the general combining ability because the other half of the genetic materials in the embryos are contributed by many different pollen parents rather than a single parent, as in a full sib cross. In such cases, the markers associated with the QTLs could be more informative in the selection made at the family level.

Marker-assisted selection for disease resistance is probably different from MAS for other traits and is likely to be complicated (Nance *et al.* 1992). The expression of disease resistance in hosts not only depends on the genetics of the host but also on the associated pathogen. The strong interaction between rust and pathogen can affect the outcome of MAS. In this study, it was shown that QTLs for resistance to the two different WGR sources differed in both the magnitude of effect, and in their positions on the linkage maps, suggesting that the variability of virulence of WGR should be considered when the MAS is utilized.

A major goal of plant breeding is to isolate genes governing the traits of interest and to manipulate these genes as needed. The linkage analysis between molecular markers and target genes is a starting point toward this goal. Once the associations between molecular markers and the target genes are identified, the genes can eventually be isolated

and cloned using techniques such as chromosome walking (Bender *et al.* 1983) or chromosome jumping (Poustka *et al.* 1987). With the identification of QTLs in this study completed, the isolation and cloning of genes conferring resistance to WGR in lodgepole pine is a main focus of our future research, which will eventually lead to the effective manipulation of the host and the rust.

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Table 5-1. Putative QTLs for resistance to western gall rust in lodgepole pine family A00588

Trait/ QTL[a]	Linkage group	QTL[b] Marker interval	LOD position	LOD peak	Support interval	% variation explained[c]	Weight[d]
RWGRA							
QTL I	9	Ufsj-1710-Uttt_1140	0.0	8.30	Ufft-1920-OPAeb-760+10	35.6	-0.418
QTL II	9	Ulbz_2255-Ufrb-1260	16.0	5.60	2+Ulbz_2255-Utjb-1710+22	32.8	-0.408
Two-QTL model : Total phenotypic variance explained 40.2%, LOD: 9.63							
RWGRM							
QTL III	9	Ufft_1920-Uljz-1425	2.0	3.86	Ufft_1920-Ulbz_2255+14	23.2	-0.427

[a] RWGRA, Resistance to western gall rust from Alberta sources;
RWGRM, Resistance to western gall rust from Manitoba sources.

[b] cM distance from leftmost marker of interval, indicating the most likely QTL position corresponding to LOD peak.

[c] Percentage of phenotypic variance explained by the QTL as estimated by MAPMAKER/QTL.

[d] The QTL effect, as measured by the derivation of mean between the group with QTL and the group without the QTL.

Table 5-2. Putative QTLs for resistance to western gall rust in lodgepole pine family A01013

Trait/ QTL[a]	Linkage group	Marker interval	QTL[b] position	LOD peak	Support interval	% variation explained[c]	Weight[d]
RWGRA							
QTL I	1	Urbw_725-Utrz-855	20.0	4.04	18+ Urbw_725-Utrz-855+24	82.6	-0.726
QTLII	7	Ufse-635-Ursz-1540	34.0	8.05	10+ Ufse-635-Ursz-1540+26	86.4	-0.712
QTLIII	10	Ult-820-Ueee-1035	22.0	6.35	10+ Ult-820-Ueee-1035+12	86.4	-0.709
QTLIV	10	OAee-1375-Urbw-800	10.0	9.37	2+OAee-1375-Urbw-800+26	86.5	-0.689
QTLV	11	Utrs-730-Uttt-1140	18.0	6.95	10+Utrs-730-Uttt-1140+26	84.3	-0.612
Five-QTL model : Total phenotypic variance explained 87.4%, LOD: 10.63							
RWGRM							
QTL VI	10	Ulte-2030-Uwzf-800	20.0	4.25	14+ Ulte-2030-Uwzf-800+16	81.9	-0.690
QTL VII	10	OAee-1375-Urbw-800	14.0	6.00	10+ OAee-1375-Urbw-800 +18	82.3	-0.701
QTLVIII	13	Ursw-885-Urfb_1140	16.0	6.25	10+ Ursw-885-Urfb_1140+20	82.6	-0.704
QTLIX	16	OPBes-620-Ufwf-505	18.0	4.41	14+ OPBes-620-Ufwf-505+20	81.7	-0.690
Four-QTL model : Total phenotypic variance explained 84.2%, LOD: 7.63							

[a] RWGRA, Resistance to western gall rust from Alberta sources;
RWGRM, Resistance to western gall rust from Manitoba sources.

[b] cM distance from leftmost marker of interval, indicating the most likely QTL position corresponding to LOD peak.

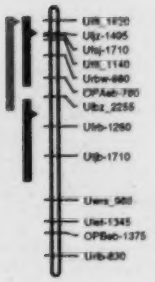
[c] Percentage of phenotypic variance explained by the QTL as estimated by MAPMAKER/QTL.

[d] The QTL effect, as measured by the derivation of mean between the group with QTL and the group without the QTL.

Table 5-3. Putative QTLs for resistance to western gall rust in lodgepole pine family A01754

Trait/ QTL[a]	Linkage group	Marker interval	QTL[b] position	LOD peak	Support interval	%variation explained[c]	Weight[d]
RWGRA							
QTL I	13	Ufsj-1360-Ulbe-1715	12.0	4.96	8+ Ufsj-1360-Ulbe-1715+20	87.3	-0.709
RWGRM							
QTL II	3	Ufrb_1330-Ulse_1540	26.0	3.26	6+ Ufrb_1330-Ulse_1540	81.5	-0.650

[a] RWGRA, Resistance to western gall rust from Alberta sources;
RWGRM, Resistance to western gall rust from Manitoba sources.
[b] cM distance from leftmost marker of interval, indicating the most likely QTL position corresponding to LOD peak.
[c] Percentage of phenotypic variance explained by the QTL as estimated by MAPMAKER/QTL.
[d] The QTL effect, as measured by the derivation of mean between the group with QTL and the group without the QTL.



Group 9

Resistance to western gall rust from Alberta sources, RWGRA
Resistance to western gall rust from Manitoba sources, RWGRM

Figure 5-1. Putative QTLs for resistance to western gall rust in lodgepole pine family A00588. The arrow keys and bars indicated the most likely positions of the QTL and their corresponding support intervals at LOD of 1.0.

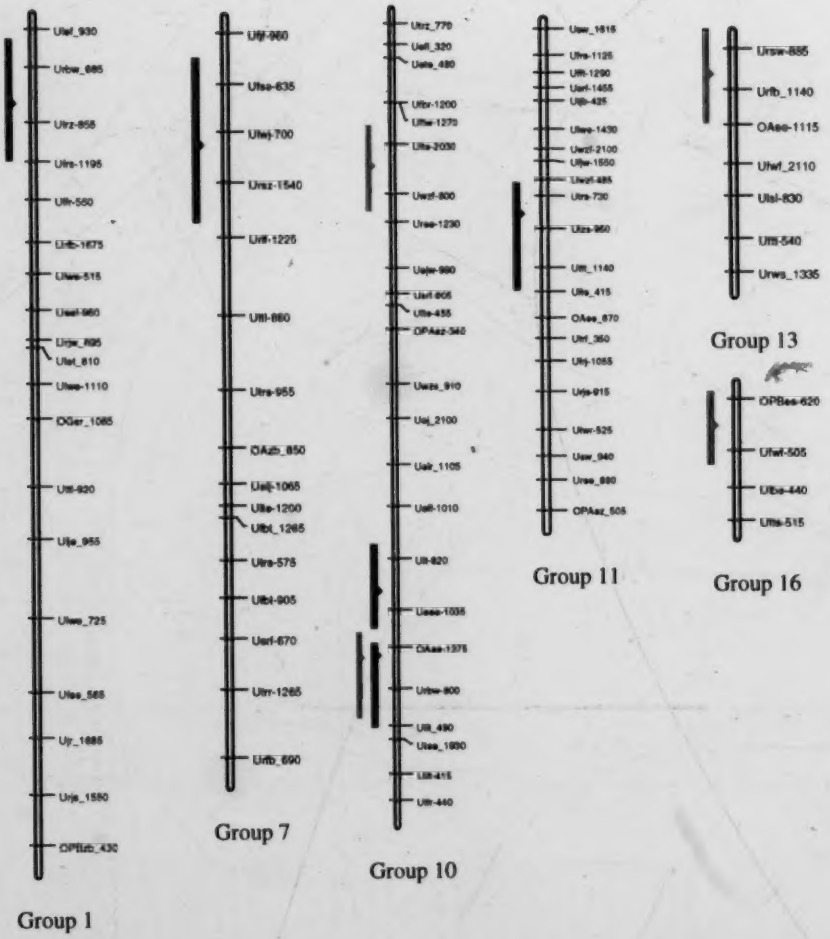
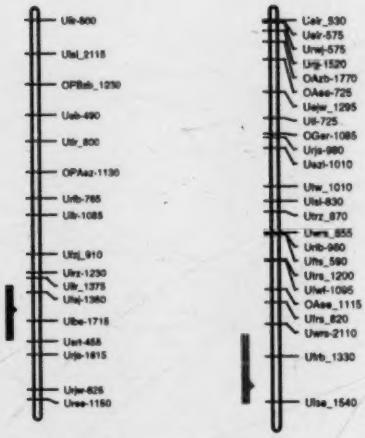


Figure 5-2. Putative QTLs for resistance to western gall rust in lodgepole pine family A01013. The arrow keys and bars indicated the most likely positions of the QTL and their corresponding support intervals at LOD of 1.0.



Resistance to western gall rust from Alberta sources, RWGRA
Resistance to western gall rust from Manitoba sources, RWGRM

Figure 5-3. Putative QTLs for resistance to western gall rust in lodgepole pine family A01754. The arrow keys and bars indicated the most likely positions of the QTL and their corresponding support intervals at LOD of 1.0.

CHAPTER 6 GENERAL DISCUSSION, CONCLUSION AND IMPLICATIONS

6.1 INTRODUCTION

It is widely accepted that plant hosts and their associated pathogens, interacting over evolutionary time, co-evolve complementary genetic systems that regulate the host and the pathogen populations in dynamic balance with one another (Person 1959; Day 1974; van der Plank 1975; Harlan 1976). Endemic disease levels are the expected outcome of coevolution between a host and its pathogen. Disease epidemics occur when the balance is disturbed (Nance *et al.* 1992).

Western gall rust (WGR) is a major fungal disease in lodgepole pine (Hiratsuka 1987). As an obligate parasite without alternative hosts, WGR infects lodgepole pine directly and results in a great economic loss by killing the trees or reducing the wood quality (Powell and Hiratsuka 1973; van der Kamp and Spence 1987; Bella and Navratil 1988). As the management of lodgepole pine plantations becomes intensive, effective control of the disease in these plantations is becoming a primary concern.

This study was designed to examine the genetic variability of WGR, evaluate the resistance of lodgepole pine against genetically different WGR spore sources and identify quantitative trait loci for resistance to the rust. The data presented in this thesis will enhance our understanding of virulence variability in the rust, resistance variation in the host, and the genetic basis governing the interaction between the rust and the host. This chapter reviews the general results reported in this study and outlines some implications of the results in the effective manipulation of both western gall rust and lodgepole pine.

6.2 GENETIC VARIABILITY OF WESTERN GALL RUST FUNGUS

Genetic variability of WGR among different geographic collections was commonly observed previous to this study (Tuskan *et al.* 1990; Vogler *et al.* 1991; Sun *et al.* 1995; Hubbes and Lin 1995). The most important finding in this study was the distinct RAPD profiles observed between the isolates from lodgepole pine and the isolates from jack pine, which might suggest host specificity in WGR. The isolates of jack pine origin exhibited significant variability in their RAPD profiles among locations, with an east-west trend of decreasing affinity. In contrast, uniformity of RAPD pattern was found among the WGR collections of lodgepole pine origin.

Genetic variability between the rust collections could be indicative of virulence differences, which has implications in the rust-host pathosystem study. Selection of genetically different isolates will increase the effectiveness in examination of rust-host interaction. Greenhouse evaluation of resistance for three lodgepole pine families in this study showed significant spore source effect. Rust isolates collected from Manitoba, of jack pine origin, tended to be more virulent to the Alberta lodgepole pine than the isolates from Alberta of lodgepole pine origin.

Compared to the genetic variability in isolates of jack pine origin, uniformity of RAPDs among the rust collections of lodgepole pine origin might suggest less selection pressure from lodgepole pine. Other observations showed a trend that lodgepole pine is more susceptible than jack pine to WGR (Wu *et al.* 1996; Yang *et al* 1997). Different selection pressure from the hosts could lead to divergent genetic structures in the rust populations. However, the interpretation of homogeneity of RAPDs in WGR isolates sampled from lodgepole pine should be viewed with caution. Limited sample size and the

dominant nature of RAPD markers might contribute, to some extent, to the observed results. Thus, a detailed investigation of genetic variability among isolates of lodgepole pine origin would be beneficial for a thorough understanding of the genetic structure of the rust and the coevolution process between the rust and the host.

6.3 RESISTANCE OF LODGEPOLE PINE TO WESTERN GALL RUST

Resistance of three lodgepole pine half-sib families from Alberta, family A00588, family A01013 and family A01754, was evaluated against two WGR sources in this study. The two WGR isolates were collected from Alberta (lodgepole pine origin) and Manitoba (jack pine origin) and showed distinct RAPD profiles.

Variance analysis of infection ratings indicated significant family effect, rust source effect, and interaction between family and rust source. Overall, family A01013 was less infected by WGR, with an infection rating score of 1.825 using the 0-5 scale (non-infection to complete gall) while family A00588 was the most susceptible to WGR, with an infection rating score of 2.738. Family A01754 was ranked between them, with an infection rating score of 2.326.

The two rust sources demonstrated different levels of virulence. In general, the isolate of jack pine origin tended to be more virulent than the isolate of lodgepole pine origin. On average, the three lodgepole pine families had an infection rating score of 2.559 against the rust isolate of jack pine origin and an infection rating score of 2.056 was observed against the rust isolate of lodgepole pine origin.

The three lodgepole pine families had similar infection ratings against the rust source of lodgepole pine origin. In contrast, significant difference in response to the rust of jack

pine origin was detected. Family A00588 was the most susceptible to the rust of jack pine origin, with an infection rating score of 3.517 using 0-5 scale (non-infection to complete gall). Family A01013 was the most resistant to the rust of jack pine origin, with an infection rating score of 1.618. Family A01754 was intermediate, with an infection rating score of 2.541. The great variation in resistance to WGR in the three lodgepole pine families against the two genetically different WGR isolates implies the potential of selection for resistance to WGR in lodgepole pine.

The greater divergence of resistance among the three lodgepole pine families against the rust isolates collected from Manitoba might also suggest a higher host selection pressure imposed by the rust of jack pine origin. Thus, introduction of exotic rust spores could be risky to local host populations. The significant interaction between the rust and the host reported in this study should also be considered in manipulation of both the rust and the host.

6.4 GENOMIC MAP AND QUANTITATIVE TRAIT LOCI FOR RESISTANCE TO WESTERN GALL RUST IN LODGEPOLE PINE

The genetic linkage map of each family was constructed by cosegregation analysis among the RAPD markers amplified from their haploid megagametophyte DNAs. Linkage map of family A00588 has 225 RAPD markers in 16 groups, and covers 3517.5 cM. Linkage map of family A01013 has 172 RAPD markers in 16 groups, and covers 3496.2 cM. Linkage map of family A01754 has 243 RAPD markers in 17 groups, and covers 3398.6 cM. On average, the linkage maps of the three lodgepole pine families span about 95.7% of their genomes with a distance of 16.6 cM between two adjacent markers.

Quantitative trait loci (QTL) for resistance to two different WGR isolates were identified and mapped by the half-sib analysis for each of the three half-sib lodgepole pine families based on their genetic linkage maps. The three half-sib lodgepole pine families were selected because they exhibited a large phenotypic variance in resistance to WGR in long-term field observations and greenhouse screening. The greenhouse evaluation in this study confirmed the great variation in resistance to WGR among the individuals of each half-sib family, which held the promise that the QTLs for resistance to WGR could be detected by the half-sib analysis.

Fourteen QTLs for resistance to WGR were identified in total for the three lodgepole pine families in this study. Among them are eight QTLs for resistance to WGR of lodgepole pine origin from Alberta and six for the rust of jack pine origin from Manitoba. The QTLs identified in this study each explained the total phenotypic variance in resistance to WGR from a range of 23.2% to 87.3%. QTLs with larger effects were mostly found in family A01013 and family A01754. In family A01013, five QTLs for resistance to the rust from Alberta and four QTLs for resistance to the rust from Manitoba accounted for more than 80% of the total phenotypic variance in resistance. In family A01754, two QTLs controlled 87.3% and 81.5% of the total phenotypic variance in resistance to the rust from Alberta and from Manitoba respectively.

QTLs with relatively small effects were only detected in fanily A00588. Two QTLs for resistance to the rust from Alberta accounted for 35.6% and 32.8% of the total phenotypic variance in resistance. The third QTL, which was responsible for resistance to the rust from Manitoba, explained 23.2% of the total phenotypic variance in resistance.

Kojwang and van der Kamp (1991) proposed that single major resistance may play a role in the western gall rust-lodgepole pine pathosystem, but polygenic resistances of smaller effect predominate. In this study both QTLs with large effects and small effects were detected. However, QTLs with large effects and small effects were not present in the same family. In family A00588, the majority of its half-sib progeny showed complete infections or partial infections by the rust, indicating the family has accumulated resistance genes with only small effects against the two rust isolates. In contrast, family A01013 seems to possess several resistance genes with large effects, resulting in a substantial proportion of non-infections among its half-sib progeny. In family A01754, only two QTLs with large effects were detected, which might suggest the family has accumulated a few major resistance genes against the two rust sources.

The majority of QTLs of large effect identified in this study may not contrast with the hypothesis proposed by Kojwang and van der Kamp (1991) that polygenic resistances of smaller effect predominate in the lodgepole pine-western gall rust pathosystem. The selection of the three half-sib lodgepole pine families with large variations in resistance in this study might only favor the QTLs of large effect to be detected. The limited sample sizes might also make the QTLs of small effect undetectable.

Each of nine QTLs detected in family A01013 has a large effect, which suggests one QTL could represent all the QTLs. However, the nine QTLs in family A01013 were found in six different linkage groups. It seems that the QTLs are related to each other and appear as a complex, although they are not physically linked, because gametic disequilibria between independent loci have been observed in lodgepole pine (Yang and Yeh 1993). However, it should be addressed that given the fact that the progeny size was

30 and 45 in this study, and a LOD score of 3.0 was used in map construction, we anticipate the inclusion of spurious QTLs, especially those with LOD score below 4.90 (corresponding to a false positive rate of approximately 1×10^{-3}).

QTLs for resistance to the rust from Alberta and the QTLs for resistance to the rust from Manitoba showed associations in this study in terms of their positions on the genetic linkage maps. However, without further analysis it is still difficult to tell whether these QTLs are the same. Although some of them are only a few cM apart on the same linkage map, they could be different sections of DNA since 1 cM in conifers could be 4000 kilobases or more (Neale and Williams 1991).

One of the potential implications of identifying QTLs for commercially important traits is marker-assisted selection (MAS) (Neale and Williams 1991; Dudley 1993; Staub *et al.* 1996). The quantitative traits of interest could be improved with the aid of selection of the genetic markers linked to the QTLs. In this study, eight QTLs were identified for resistance to WGR of lodgepole pine origin from Alberta. Six QTLs were for resistance to the rust of jack pine origin from Manitoba. Thirty-one RAPD markers were found within the 1.0 LOD support intervals of these QTLs. These RAPD markers could be tested for their potential uses for MAS in the manipulation of both the rust and lodgepole pine.

RAPD markers linked to the QTLs could also be used in studies of co-evolution of WGR and lodgepole pine. The distribution of the QTL-linked markers in populations of the host might shed some light on the co-evolutionary process between the rust and the host. Further studies of the relationship between the distribution of QTL-linked markers

and the genetic variability in the rust may also increase the understanding of the rust-host pathosystem.

One of the goals in genetic studies is to isolate and characterize the genes of interest. Identification of QTLs is a starting point toward this goal. With the positions of QTLs of interest defined on the chromosomes, the genes of interest can be eventually isolated and cloned using techniques such as chromosome walking (Bender *et al.* 1983) or chromosome jumping (Poustka *et al.* 1987). Only when the resistance genes in lodgepole pine are cloned and their expressions well understood would the manipulation of both the rust and the host become effective in control of the disease.

6.5 FUTURE STUDIES RECOMMENDED

This study examined the geographic variability of WGR fungus, investigated the association between the resistance of lodgepole pine and the genetically different WGR isolates, and identified fourteen quantitative trait loci (QTL) for resistance to WGR in lodgepole pine. The results reported in this thesis provide useful information for understanding the rust-host pathosystem. However, many questions are still unanswered. Consequently, additional studies are still needed.

Western gall rust exhibited distinctive genetic differentiation between the collections of lodgepole pine and jack pine hosts, suggesting its strong association with the hosts. However, the coevolution of WGR with its host is still poorly understood, and it is also of interest to discern the relative roles of historical factors, ecological factors and contemporary factors such as gene flow, genetic draft, mating system and selection in the present geographic diversity of the rust.

Extensive investigation of resistance response involving a wide range of host populations against genetically divergent WGR isolates are still essential to effective selection and breeding of resistant host stocks to the rust. Also a closer examination of interaction of the host and the rust will be beneficial for the understanding of coevolution process of the rust and the host.

Quantitative trait loci for resistance to western gall rust in lodgepole pine have been identified and mapped in this study, and consequently RAPD markers that are closely linked to these QTLs could be tested for their potential uses in marker-assisted selection. The test can be carried out using another set of samples originated from the same family from which the QTLs were identified, or using other populations to exploit common QTLs for a variety of hosts. It is also of interest to examine the distribution of those QTL-linked RAPD markers among populations of lodgepole pine because the pattern of the marker distribution might reflect the evolutionary mechanisms of the rust associated with its hosts.

Localization and cloning of resistance genes could be initiated in lodgepole pine with availability of the map positions of the QTLs identified in this study and their linkage relationships with the RAPD markers. Chromosome walking (Bender *et al.* 1983) or chromosome jumping (Poustka *et al.* 1987) could be used to determine the positions of QTLs on the chromosomes and lead to the cloning of the genes. Once the resistance genes are successfully cloned, testing of the many hypotheses regarding the host-pathogen system such as gene-for-gene interaction, models of resistance and pleiotropic effects of resistance genes should be straightforward.

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